CHLOROPLAST GENE EXPRESSION DURING LEAF DEVELOPMENT

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by

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DECLARATION

I declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work is my own, except where specifically acknowledged.

Jane Silverthorne

SUMMARY

The growth and development of the primary leaf of spinach (*Spinacia oleracea*) have been characterized with respect to both physical and biochemical parameters. Excised primary leaves incorporate [^{35}S] methionine into a number of chloroplast polypeptides. The ratio of incorporation of isotope into two chloroplast polypeptides, the large subunit of Fraction I protein and a thylakoid polypeptide (peak D), decreases during leaf development in whole leaves. This changing pattern of incorporation is also observed in isolated chloroplasts where these two polypeptides are the major products of protein synthesis. Chloroplast RNA prepared from developing leaves was translated in a cell-free protein synthesizing system prepared from rabbit reticulocytes to yield full-length large subunit and peak D polypeptides. The fidelity of translation of these two polypeptides was checked by partial protease digestion. Changes in the synthesis of the large subunit of Fraction I protein and peak D in developing leaves are reflected in changes in the amount of translatable mRNA for these two polypeptides. These findings suggest that the expression of chloroplast genes during development is at least partially controlled at the transcriptional level.

Abbreviations

A	adenosine base
A_X	absorbance at wavelength X
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
C	cytosine base
CF_1	chloroplast coupling factor 1
CF_O	chloroplast coupling factor membrane portion
Ci	Curie (3.7×10^{10} disintegrations per second)
CPI	chlorophyllin I
CPII	chlorophyllin II
cpm	counts per minute
ct DNA	chloroplast DNA
DCCD	dicyclohexyl carbodiimide
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
EDTA	ethylene diamine tetraacetic acid
EF	internal thylakoid fracture face
EGTA	ethylene glycol tetraacetic acid
ES	internal thylakoid surface
G	guanosine base
g	gravitational force
GTP	guanosine triphosphate
Hepes	N- 2- hydroxyethyl piperazine-N-2-ethane sulphonic acid

LHCP	light-harvesting chlorophyll a/b protein complex
mA	milliampere
MDMP	2-(4-methyl-2,6-dinitroanilino)-N-methyl propionamide
Mes	2-(N-morpholino) ethane sulphonic acid
mRNA	messenger RNA
M.wt.	molecular weight
oligo (dT)	oligo deoxythymidylic acid
p680	PSII reaction centre chlorophyll
p700	PSI reaction centre chlorophyll
pH	$\log_{10}$ hydrogen ion concentration
PMSF	phenylmethyl sulphonyl fluoride
poly(A)	polyadenylic acid
POPOP	1,4-bis-(5-phenyloxazol-2-yl) benzene
PPO	2,5-phenyl oxazole
PF	external thylakoid fracture face
PS	external thylakoid surface
PSI	photosystem I
PSII	photosystem II
RBPcase	ribulose bisphosphate carboxylase
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylene diamine
TMV	tobacco mosaic virus
Tricine	N-tris (hydroxymethyl) methyl glycine
Triton X-100	octyl phenoxy polyethoxyethanol

SECTION I - LITERATURE REVIEW

II. INTRODUCTION

Photosynthetic plant cells contain a class of organelle, the chloroplast, which functions to convert solar energy into chemical energy by the process known as photosynthesis (Halliwell, 1978). It has been known since 1962 that chloroplasts contain deoxyribonucleic acid (DNA) and a protein-synthetic machinery based on 70S ribosomes which together comprise a discrete genetic system (Boulter et al 1972; Ellis, 1977). However, although chloroplasts have the potential for complete genetic autonomy, it has become clear that this autonomy is only partial. Many chloroplast polypeptides are synthesized in the cytoplasm on 80S ribosomes and subsequently enter the chloroplast (Börner, 1972; Ellis et al, 1973).

The successful isolation of intact chloroplasts retaining the ability to synthesize chloroplast polypeptides in vitro has allowed the study of the function of chloroplast ribosomes (Blair and Ellis, 1973; Ellis, 1977). Higher plant chloroplasts isolated from a number of species synthesize two major polypeptides in vitro (Ellis, 1977). The first of these, a soluble polypeptide, is the large subunit of ribulose bisphosphate carboxylase, the enzyme which catalyzes the first step in the fixation of CO_2 in the Calvin cycle (Blair and Ellis, 1973). The second polypeptide is thylakoid-bound and has an apparent molecular weight of 32,000 (Eaglesham and Ellis, 1974). Its function is unknown.

In more recent studies, the emphasis has been on the determination of which genes are encoded in the chloroplast DNA (Bedbrook and Kolodner, 1979) and the expression of these genes during chloroplast development (Bradbeer, 1975). Chloroplast DNA is now known to contain

the genes for both the large subunit of ribulose bisphosphate carboxylase and the 32000 molecular weight thylakoid polypeptide (Kung, 1977; Bedbrook et al, 1979; Bedbrook et al, 1978). The expression of these genes during the development of the spinach primary leaf under normal growth conditions forms the main aim of this thesis.

The literature review is intended to set the scene for the study of chloroplast development with respect to both chloroplast structure and synthesis. Since the approach adopted in this study (to be outlined in Section I5) is largely biochemical, it is important to relate such information to the known structure and function of chloroplasts in vivo. Section I2 is concerned with the structure and synthesis of chloroplasts. The development of chloroplasts from etioplasts and proplastids is outlined in Section I3. Since a range of translational systems is available to assay messenger ribonucleic acid (mRNA) in developing systems, the advantages and disadvantages of the most widely-used systems are reviewed in Section I4. The aims and approaches adopted in this study are set out in Section I5.

I2. THE SYNTHESIS OF CHLOROPLASTS

A. The structure of the mature chloroplast.

(i) The chloroplast envelope.

Chloroplasts are lens-shaped green bodies, 6-8 μm in diameter, which are found in the cytoplasm of green plant cells (Kirk and Tilney-Bassett, 1978). The internal structure of the chloroplast is divided from the cytoplasm by a double membrane termed the chloroplast envelope. Chloroplast envelopes have been purified from bean (Mackender and Leech, 1970), spinach (Poincelot, 1973; Douce et al, 1973; Poincelot and Day, 1974; Pineau and Douce, 1974), pea (Joy and Ellis, 1975) and oat (Wellburn and Cobb, 1974). Such preparations appear as double and single membrane-bounded vesicles when examined under the electron microscope.

A number of enzymic activities are associated with purified envelope preparations. Sabnis et al (1970) have located a magnesium-dependent adenosine triphosphatase (ATPase) activity between the two envelope membranes of pea tendril chloroplasts. This activity is quite distinct from the latent calcium-dependent ATPase of thylakoids and is therefore a suitable marker for envelope purity. Chloroplast envelope fractions have also been shown to contain phosphatidate phosphohydrolase (Joyard and Douce, 1979) and adenylate kinase activities (Murakami and Strotmann, 1978). The envelope is the site of synthesis of galactolipids (Douce, 1974) and galactosyldiacylglycerols (Van Besouw and Wintermans, 1979). Most of the protein-bound hexosamine in chloroplasts is bound to the chloroplast envelope (Racusen and Poincelot, 1976).

Purified envelopes do not contain chlorophyll *a* or chlorophyll *b* but appear yellow because they contain carotenoids, especially

violoxanthin (Douce and Benson 1973; Sieferman-Harms et al, 1978). In spinach chloroplasts, this composition is unique and perhaps functions to protect the chloroplast against harmful photooxidation (Jeffrey et al, 1974).

The lipid and fatty acid compositions of chloroplast envelopes are qualitatively similar to the thylakoids of the same species, although there are some quantitative differences. In bean, both thylakoids and envelopes contain similar amounts of phosphatidyl glycerol but neither membrane contains any phosphatidyl ethanolamine (Mackender and Leech, 1974). In this species, the envelope fraction contains considerably more phosphatidyl choline than the thylakoid fraction. Spinach envelopes have been shown to contain many minor lipid components which are not detectable in thylakoids; for example, cebroside, steryl glycerol and steryl esters (Poincelot, 1973).

The lipid composition of the chloroplast envelope may not be fixed but may vary according to the conditions. For example, chloroplast envelopes of salt-adapted barley contain a reduced amount of galactolipid when compared to those from non salt-adapted plants. This situation can be reversed when the plants are returned to normal conditions (Muller and Santarius, 1978).

The protein composition of chloroplast envelopes has been extensively studied in spinach (Mendiola-Morgenthaler and Morgenthaler 1974; Pineau and Douce, 1974; Sprey and Laetsch, 1975; Flugge and Heldt, 1976, 1977). The pattern of envelope polypeptides on SDS polyacrylamide gels is quite distinct from that observed for thylakoid or stromal polypeptides prepared from the same tissue. At least 25 polypeptides have been resolved from pea envelopes; two of these polypeptides are synthesized on chloroplast ribosomes (Joy and Ellis, 1975). The polypeptide of apparent molecular weight 29000

observed in gel patterns of spinach envelope polypeptides (Pineau and Douce, 1974) may be identical with the polypeptide associated with phosphate translocation (Flugge and Heldt, 1976, 1977).

The movement of molecules in and out of the chloroplast is to some extent determined by the chloroplast envelope. This subject has been reviewed by Heber (1974) and Heldt (1976). Generally, the outer membrane of the envelope is freely permeable to small molecules (Heldt and Sauer, 1971) whilst the inner membrane is relatively impermeable (Gimmler et al, 1974). The inner envelope membrane is the site of several specific translocators (Poincelot, 1975). Heldt and Rapley (1970 a,b) have described specific translocators for phosphate and dicarboxylic acids.

(ii) The stroma.

The internal space of the chloroplast contains a granular, electron-dense phase known as stroma, and an array of membranes which constitute the thylakoids. The stroma contains ribosomes, transfer RNA (tRNA) species, amino acid activating enzymes (Franki et al, 1965) and various intermediary metabolites (Givan and Leech 1971; Givan and Harwood, 1977).

The enzymes of the Calvin cycle are located in the stroma (Trebst et al, 1958) and comprise most of the water soluble, stromal protein (Kirk and Tilney-Bassett, 1978). The majority of this protein is accounted for by the enzyme ribulose 1,5 bisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing) E.C. 4.1.1.39); the proportion of the stromal protein accounted for by this enzyme changes with leaf age (Dorner et al, 1957).

Ribulose bisphosphate carboxylase was first isolated from spinach leaves by Wildman and Bonner (1947), and was given the name Fraction I protein because of its fractionation on Tiselius moving boundary

electrophoresis. This fraction contained a protein of sedimentation coefficient 18S which accounted for about 50% of the leaf soluble protein. Weissbach et al (1956) later showed that Fraction I protein has the enzymic activity of ribulose bisphosphate carboxylase. Since some bacteria contain an enzymic activity associated with a protein of different properties from that of algae and higher plants, Fraction I protein will be used to describe the protein isolated from higher plants and photosynthetic algae only (Ellis, 1974).

Native Fraction I protein has a molecular weight of between 5.1 and 5.6 x 10^5, depending on the plant species (Kawashima and Wildman, 1971b; Akazawa et al, 1972; McFadden et al, 1975). Fraction I protein is composed of two dissimilar subunits which are generally described as large subunit (LSU) and small subunit (SSU). These subunits can be dissociated using sodium dodecylsulphate (SDS) (Rutner and Lane, 1967), urea (Moon and Thompson, 1969; Sugiyama and Akazawa, 1970) or high pH (Kawashima and Wildman, 1971b). The molecular weights of the two subunits have been estimated as 52000-55000 for LSU and 11000-16000 for SSU (Gray and Kekwick, 1974; Iwanij et al, 1974; Ellis, 1976; Moon and Thompson, 1969; Rutner, 1970). The variability of these estimates is partially a reflection of the methods used (Ellis, 1974). These subunits are not joined by disulphide bonds because reducing agents are not required to effect separation of LSU and SSU (Kawashima and Wildman, 1970).

There is evidence that ribulose bisphosphate carboxylase molecules in some organisms do not contain both LSU and SSU. McFadden (1973) has reviewed the evolutionary aspects of these differing subunit compositions and classified the organisms into three groups. Group I, which includes higher plants and green algae, contain enzymes having both LSU and SSU, whereas Group II and III organisms contain a single subunit of similar size to LSU. Ribulose

bisphosphate carboxylase molecules from Rhodospirillum rubrum and Chlorobium thiosulphatophilum fall into these latter categories (Tabita and McFadden, 1974; Tabita et al, 1974.)

The number of subunits peroligomer molecule in native Fraction I protein from spinach (Rutner, 1970), Euglena (Rabinowitz et al, 1975) and tobacco (Baker et al, 1975) is probably 8 LSU molecules and 8-10 SSU molecules. There is some uncertainty over the number of SSU molecules because of the errors in the estimate of the molecular weight of this subunit. The most probable structure of Fraction I protein from electron microscopic studies (Kawashima and Wildman, 1970; Baker et al, 1975; Steer et al, 1968; Howell and Moudrianakis, 1965) and optical diffraction (Baker et al, 1975, 1977) is a cube of LSU molecules with SSU molecules attached to the outside of this structure. Under the electron microscope, Fraction I protein is an isodiametric molecule, 10-12 nm across, with a central hole. Until recently, only tobacco Fraction I protein was available in a crystalline form (Kawashima and Wildman, 1971a) suitable for optical studies; the recent successful crystallization of Fraction I protein from spinach (Johal and Bourque, 1979) and barley (Stroback and Gibbons, 1976) should enable further comparative studies.

The evolutionary conservation of the amino acid sequences of LSU and SSU polypeptides has been studied by a number of techniques. On the basis of amino acid compositions (Iwanij et al, 1974; Rabinowitz et al, 1975; Rutner and Lane, 1967), tryptic peptides analyses (Kawashima and Wildman, 1971b; Kawashima et al, 1971; Kung et al, 1974) and antigenic determinants (McFadden, 1973; Gray and Kekwick, 1974) the structure of LSU appears to be highly conserved whilst the structure of SSU is variable. For example, antiserum raised against spinach LSU will cross-react against Fraction I protein from blue-green algae (Takabe et al, 1976). The large subunit carries both

carboxylase and oxygenase activities whilst SSU may have a regulatory function (Nishimura and Akazawa, 1973, 1974); the relative conservation of the amino acid sequences may reflect these functions. Until complete amino acid sequences are available for LSU and SSU polypeptides from a number of species, it is not possible to say which regions are conserved. At present only the SSU sequence in spinach has been completely determined (Martin, 1979).

Isoelectric focusing of LSU and SSU polypeptides on polyacrylamide gels has revealed that there are several charge variants of each molecule; there are three LSU variants and up to four SSU variants (Kung et al, 1974). LSU has three charge variants in all the species so far examined and these variants have identical tryptic maps. The cause of the charge variation is unknown (Wildman et al, 1975). The isoelectric variants have been used successfully in the genetic studies of the inheritance of the genes encoding LSU and SSU (Section I2B).

Fraction I protein catalyzes two reactions:-

1. CO_2 + D-ribulose 1,5 - bisphosphate + H_2O →
   2[3-phospho-D-glycerate].

2. O_2 + D-ribulose - 1,5 - bisphosphate →
   3-phospho-D-glycerate
   + phosphoglycolate.

Reaction 1 is the carboxylation reaction of the Calvin cycle (Halliwell, 1978); reaction 2 has been implicated in photorespiration (Lorimer et al, 1973), the process whereby carbon fixed in reaction 1 is lost as CO_2 by a series of light-dependent reactions (Chollet and Ogren, 1975). This subject has been reviewed recently by Chollet and

Ogren (1975) and Jensen and Bahr (1977). There is some controversy as to whether Fraction I protein contains both these enzymic activities, or whether the oxygenase is a minor contaminant which copurifies with Fraction I protein. Bränden (1978) has claimed to separate ribulose bisphosphate carboxylase activity from the oxygenase activity in parsley extracts. He correlates a blue colour and copper EPR signal only with the oxygenase.McCurry et al (1978) carried out a similar preparation with parsley but could not separate the activities. Crystals of purified spinach Fraction I protein do not contain any detectable copper, although it may be argued that this finding is a reflection of the source of tissue and the method of preparation (Johal and Bourque, 1979). On the other hand, it seems likely that the carboxylase and oxygenase activities are properties of the same protein because they both have a catalytic site on separated LSU (Nishimura and Akazawa, 1974; Takabe and Akazawa, 1973) and both activities are inhibited by cyanide (Marsho and Kung, 1976) and 2-carboxyribitol 1,5-bisphosphate (Ryan and Tolbert, 1975). In fact, the same active site may be involved in both activities (Lorimer and Andrews, 1973; Jensen and Bahr, 1977).

(iii) The internal membranes.

The internal region of the chloroplast is traversed by a network of flattened vesicles which have been given the name thylakoids by Menke (1962). Recent reviews by Anderson (1975), Boardman et al (1978), Arntzen (1978) and Trebst (1974) have considered the molecular architecture of these membranes. The thylakoid membranes may be divided into two classes according to their arrangement: the granal lamellae which are formed into stacks or grana, and the stromal lamellae which interconnect the grana.

There are commonly between 5 and 20 thylakoids in a granum and between 40 and 60 grana per chloroplast, although the exact numbers depend on the species examined and the growth conditions (Kirk and Tilney-Bassett, 1978).

Recent evidence from electron microscopic and biochemical studies support the Singer model of membrane structure (Singer, 1974; Singer and Nicholson, 1972) as a model for the structure of the thylakoid membranes. In this model, the membrane consists of a lipid bilayer in which intrinsic proteins are embedded, and to which extrinsic proteins are attached. Electron microscopic studies reveal that the structure of the thylakoid membrane is asymmetric. When thylakoids are freeze fractured, the plane of fracture is probably parallel to the thylakoid plane along the hydrophobic interior (Branton, 1966). The fracture face corresponding to the inner portion of the membrane (EF face) contains large particles (17.5 nm diameter) in granal lamellae but not in stromal lamellae (Boardman et al, 1978). There are between 200 and 1300 particles per μm^2 on this face (Park and Sane, 1971). The fracture face corresponding to the outer portion of the membrane (PF face), which is complementary to the EF face, has particles of 7-11 nm embedded in it (Boardman et al, 1978) at a density of about 5000 per μm^2.

The outer thylakoid surface (PS), when negatively stained, can be seen to have large numbers of 10 nm particles scattered over its area (Howell and Moudrianakis, 1967). These particles can be removed by EDTA treatment, this treatment resulting in a loss of photophosphorylation activity. Such EDTA extracts contain an ATP ase activity which can be obtained from chloroplast coupling factor (CF_1) extracts. Chloroplast coupling factor was so named because it stimulates photophorsphorylation in subchloroplast particles

(Avron, 1963; Vambutas and Racker, 1970). When the EDTA extract is added back to the membrane, the ATPase activity and particles are restored (Lien and Racker, 1971.) Purified CF_1 particles have a diameter of about 15 nm under the electron microscope after freeze etching (Garber and Steponkus, 1974). Similar particles are visible on the PS surface of thylakoids and about 30% of these can be removed by washing with 10 mM sodium pyrophosphate; the remainder are removable with EDTA (Garber and Steponkus, 1974; Miller and Staehelin, 1976). Immunological studies have confirmed that CF_1 is attached to the outer thylakoid surface. This contrasts with the inner thylakoid surface (ES) which contains no attached particles, although the EF particles may protrude (Miller and Staehelin, 1976).

The potential identities of the EF and PF particles have, to some extent, been studied by the use of detergents. This area has been reviewed by Thornber (1975), Thornber et al, (1977), Arntzen (1978) and Thornber et al, (1979). Non-ionic detergents such as digitonin and Triton X-100, which break non-ionic interactions within membranes resolve thylakoids into particles containing lipid and several polypeptides. These complexes are photochemically active and may therefore be used to elucidate the functional organization of thylakoids (Arntzen 1978). Arntzen describes six possible complexes which are resolved by non ionic detergents:- the photosystem II (PSII) reaction centre, the light harvesting complex, the photosystem I (PSI) reaction centre, the cytochrome f-b_6 complex and the hydrophobic complex of the coupling factor (CF_o). The photosystem II reaction centre contains p680. p680 is the name given to a form of chlorophyll a which, by virtue of its position in the membrane, functions as the photoactive receptor for photosystem II. PSII also contains the light-harvesting chlorophyll a, and at

least six polypeptides with molecular weights between 27000 and 54000. In contrast, the photosystem I complex contains P700. P700 is the name given to photoactive chlorophyll *a* molecules in PSI. PSI also contains six polypeptides with molecular weights between 10000 and 74000, and a chlorophyll *a/b* ratio of at least 7.5. The light harvesting complex has no photochemical activity; it contains several polypeptides in the region of molecular weight 20000 to 25000, including the chlorophyll *a/b* binding protein.

Arntzen (1978) has proposed that the large EF particles correspond to a complex containing photosystem II and the light-harvesting apparatus. If seedlings are grown under intermittent light, the chloroplasts do not contain chlorophyll *b* and the light-harvesting complex. When such plants are greened under continuous light, chlorophyll *b* and the light-harvesting complex are synthesized, and there is a concomitant appearance of particles on the EF face of stacked lamellae (Armond *et al*, 1977). The change in particle size on the EF face during greening is consistent with the addition of light-harvesting complexes to a PSII core, the final size of the particle being a function of the amount of light-harvesting complex bound. The evidence for this assumption comes from studies of chlorophyll *b*-deficient mutants. Such a mutant of *Chlamydomonas reinhardii* does not contain the largest size class of EF particles (Goodenough and Staehelin, 1971). Similarly, chloroplast from the *Chlorina* barley mutant, which lacks a pigmented light-harvesting complex, (Henriques and Park, 1975a; Machold *et al*, 1977) have an average EF particle of 12.5 nm, instead of the 16 nm particles observed in wild-type plants (Miller *et al*, 1976).

The small PF particles may be identical with the photosystem I complex on the basis of freeze-fracture evidence (Anderson *et al*,

1973; Arntzen et al, 1972). On the basis of such evidence, Arntzen (1978) and Kirk and Tilney-Bassett (1978) have put forward models to account for the position, function and fractionation patterns of the chlorophyll protein complexes. In both models, the stacking of grana is visualized as requiring the light harvesting protein complex. Recent studies by Mullet and Arntzen (1980) and McDonnel and Staehelin (1980) have elegantly reconstructed stacked membranes in vitro using liposomes containing light-harvesting chlorophyll a/b binding protein complexes.

A more detailed fractionation pattern of thylakoids has been obtained using ionic detergents such as sodium dodecyl sulphate, sodium benzene dodecyl sulphate and lithium dodecyl sulphate (Thornber et al, 1979). Such fractionation also destroys much of the photochemical activities of the submembrane preparations, presumably because it also causes some disruption of the tertiary structure of membrane proteins (Arntzen, 1978). However, more recent rapid procedures using zwitterionic detergents have enabled isolation of complexes with some residual activity (Markwell et al, 1979). The characterization of these complexes will provide additional information on the identities of submembrane particles.

In their recent comprehensive review, Thornber et al (1979) propose that a chlorophyll protein holocomplex should be given the name "chlorophyllin" rather than chlorophyll protein, since the latter term can be used to mean a holocomplex or an apoprotein. This convention will be used throughout the following review, although it can also apply to a chlorophyll derivative.

Initially, three chlorophyll zones were obtained from Beta vulgaris thylakoids treated with sodium dodecyl sulphate or sodium dodecylbenzene sulphate and analyzed on polyacrylamide gels (Ogawa

et al, 1966; Thornber et al, 1967a). The first two zones at the top of such gels were the chlorophyllins CPI and CPII respectively; the fastest migrating zone contained lipids and pigments in a detergent complex. Some 20% of the membrane protein was found to be contained in the CPI band compared to 49% in the CPII band. The two complexes together therefore account for the major part of the membrane protein. The properties of such complexes have been extensively reviewed (Thornber, 1975; Thornber and Alberte, 1976; Boardman et al 1978; Thornber et al, 1979). A summary of the major properties is given in Table A.

The CPI chlorophyllin contains a high chlorophyll a : chlorophyll b ratio of about 7-12 (Boardman et al, 1978) when compared to CPII which has a ratio of about 1.1-1.8. There is some debate as to whether chlorophyll b is a true component of CPI. In addition to chlorophyll, both chlorophyllins contain carotenoids; CPI contains β carotene whereas CPII contains β carotene, violoxanthin, and neoxanthin, and is enriched in lutein (Ogawa et al, 1966; Thornber et al 1967b). The numbers and types of additional non-chlorophyllin components present in these complexes depends on the method of isolation (Thornber et al, 1979). CPI contains p700 as judged by light-induced absorbance changes (Bailey and Kreutz, 1969). The similarities between digitonin subfractions and CPI and CPII complexes on the basis of chlorophyll a/b ratios (Ogawa et al, 1966) and their relative enrichment patterns (Thornber et al, 1967a; Sironval, et al 1967) have led these authors to suggest that CPI contains the reaction centre of photosystem I. However, CPII is not identical with the reaction centre of PSII. The Chlorina barley mutant lacks CPII and chlorophyll b (Henriques and Park, 1975a) yet it contains both PSI and PSII activities (Thornber and Highkin, 1974). Thornber

Property	CPI	CPII
Synonyms	P_{700} chlorophyll *a* protein [1]	light-harvesting chlorophyll *a*/*b* protein [2]
Occurrence	ubiquitous in photosynthetically competent plants [3]	in all chlorophyll *b*-containing plants [1]
Activity	PSI photo oxidation and reduction [4,5]	-
Sedimentation coefficient	8.3S [6]	2.3 - 3.1S [6,7]
Molecular weight	66000-130000 [8,9,10]	22000-35000 [9,10,11]
Chlorophyll *a*:*b* ratio	7-12 [8,9,12]	> 1.1 [8,12,13]
Carotenoid composition	0.6 molecules β-carotene/ 75000 M.wt. chlorophyllin [14] lutein, neoxanthin and violaxanthin as minor components [14]	lutein, violaxanthin, neoxanthin and β-carotene, with lutein as the major component [6]
Polypeptide composition	major polypeptide 60000-70000 m.wt. upto 5 minor polypeptides of smaller size.[5,11,16,17]	major polypeptide chlorophyll *a*/*b* binding protein (m.wt. 27000-35000) + one other polypeptide 2-3000 m.wt. smaller. [9,10,15]
Chlorophyll: protein ratio	20 chlorophylls/ 70000 m.wt. chlorophyllin [5] 12 chlorophylls/ 130000 m.wt. chlorophyllin [18,19] 8-9 chlorophylls/ 66000 m.wt. chlorophyllin [16,19]	6 chlorophylls/30000 m.wt. polypeptide [19] 7 chlorophylls/30000 m.wt. polypeptide [18]

Table A Properties of CPI and CPII

[1] Thornber (1975)

[2] Thornber and Highkin (1974)

[3] Thornber et al (1979)

[4] Shiozawa et al (1974)

[5] Bengis and Nelson (1975)

[6] Thornber et al (1967b)

[7] Davis and Gross (1976)

[8] Thornber et al (1967a)

[9] Kung and Thornber (1971)

[10] Eaglesham and Ellis (1974)

[11] Machold (1975)

[12] Ogawa et al (1967)

[13] Nakamura et al (1976)

[14] Kirk and Tilney-Bassett (1978)

[15] Machold (1974)

[16] Chua et al (1975)

[17] Neilsen (1975)

[18] Remy et al (1977)

[19] Thornber et al (1977)

and Highkin have renamed CPII the light-harvesting chlorophyll a/b protein complex (LHCP) on the grounds that it is not an essential part of photosystem II function.

The amount of chlorophyll and additional pigments in chlorophyllins is dependent on the chlorophyll: detergent ratio used during membrane disruption. With decreasing ratios, more chlorophyll is released into the free pigment band. This has led Thornber et al (1979) to postulate that virtually all the chlorophyll is complexed with protein in vivo, the free chlorophyll being released during the detergent fractionation. Recent solublization procedures have virtually eliminated this free pigment but have also revealed the presence of new chlorophyllins. Some of these chlorophyllins are oligomers of CPI (Hoarau et al, 1977) or CPII (Remy et al 1977; Wessels and Borchert, 1978) whilst others are new (Delepelaire and Chua, 1979). The relationship of these chlorophyllins to membrane structure and function awaits further investigation.

Another approach to investigating the organization of thylakoid polypeptides is the selective solubilization of thylakoids using agents such as guanidine hydrochloride (Machold, 1975), chloroform-methanol and acetone (Chua et al, 1975). This approach yields information on the relative location of intrinsic and extrinsic polypeptides within the membrane. Extraction of Vicia faba thylakoids with 6M guanidine hydrochloride, an agent which removes extrinsic proteins, removes about 45% of the total membrane protein (Machold, 1975). Seventy per cent of LHCP and CPI was found to be in the membrane residue; the soluble fraction contained the CF_1 polypeptides and possibly the reaction centre of photosystem I. The major polypeptide component of LHCP, the chlorophyll a/b binding

protein, was soluble in guanidine hydrochloride only if the membrane was delipidated before extraction (Machold, 1975). Thus, the CF_1 complex is extrinsic, which is consistent with electron microscopic studies (Garber and Steponkus, 1974), whereas the LHCP (CPII) complex is mostly intrinsic.

Chloroform-methanol extraction of spinach thylakoids solubilized about a third of the total membrane protein (Henriques and Park, 1976a). The soluble polypeptides were rich in hydrophobic amino acids and more than 50% of this protein was accounted for by the chlorophyll a/b binding protein (molecular weight 25000). The major soluble (hydrophilic) polypeptides were CF_1 polypeptides and Fraction I protein. The major polypeptide of CPI in Chlamydomonas reinhardii and spinach (molecular weight 66000) is also soluble in chloroform-methanol (Chua et al, 1975).

It is evident that some portions of intrinsic polypeptides may be exposed at the surface of the membrane. Treatment of Vicia faba thylakoids with trypsin does not destroy membrane structure as seen under the electron microscope, but does increase the mobility of LHCP on SDS polyacrylamide gels (Suss et al, 1976). Similar results have been obtained using pea thylakoids, where the LHCP portion cleaved off by trypsin was shown to be required for cation-mediated granal stacking and subunit distribution (Steinback et al, 1979). The overall view of the thylakoid membrane from these studies is of a lipid bilayer containing chlorophyll-protein complexes in an asymmetric arrangement. Some protein complexes eg CF_1 are on the surface of the thylakoid in contact with stroma, while others eg CPII, are partially buried in the membrane.

Total solubilization of thylakoids by SDS has been used to study the polypeptide spectrum on SDS polyacrylamide gels. The numbers of polypeptide bands on one-dimensional gels has increased

as analytical methods have improved. Cylindrical gels resolved between 10 and 21 polypeptide bands (Hoober, 1970; Remy, 1971; Lagoute and Duranton, 1971; Machold, 1974; Eaglesham and Ellis, 1974). Electrophoresis of similar preparations on slab gels has increased this number to 33 in Chlamydomonas and 50 in spinach (Henriques et al, 1975). Recent attempts at using a two-dimensional analysis based on isoelectric focusing and SDS gel electrophoresis have revealed a more complex pattern of polypeptides (Novak-Hofer and Siegenthaler, 1977; Boschetti et al, 1978).

The structure and function of CF_1 polypeptides have recently been reviewed by Nelson (1976), Nelson (1977) and McCarty (1977). Coupling factor activity was first observed in chloroplasts by Avron (1963). Vambutas and Racker (1965) subsequently purified CF_1 and showed that it contains a latent ATPase activity. CF_1 contains 5 subunits named $\alpha, \beta, \gamma, \delta$ and ε in order of decreasing molecular weight (Racker et al, 1971). The stoichiometry of the CF_1 subunits is now generally agreed to be $2\alpha:2\beta:1\gamma:1\delta:2\varepsilon$ (Baird and Hammes, 1976; Nelson, 1976; Binder et al, 1978). The CF_1 polypeptides have been identified in chloroplast membrane preparations of Vicia faba (Suss, 1976) and spinach (Henriques and Park, 1976b). Fraction I protein is sometimes described as an extrinsic thylakoid protein although it is not clear whether binding is to specific sites or is fortuitous (Kannangara et al, 1970).

The CF_0 membrane-bound portion of the ATP synthase has recently been analyzed on polyacrylamide gels by Pick and Racker (1979) and can be reconstituted with CF_1 into functional proteoliposomes capable of catalyzing light-dependent ATP formation. The CF_0 contains several polypeptides including a 7500 molecular weight polypeptide which interacts with dicyclohexyl carbo-diimide (DCCD), and a 15500 molecular weight polypeptide of unknown function (Pick and Racker, 1979).

I2B. The informational content of the chloroplast genome.

Chloroplasts contain DNA in the form of covalently closed circles (Kolodner and Tewari 1972, 1975a; Herrmann et al 1975) about 38-45 um in circumference. Euglena gracilis chloroplast DNA (ct DNA) has a contour length of about 40 µm (Manning et al, 1972) whereas larger molecules have been observed in Chlamydomonas reinhardii (Behn and Herrmann 1977; Kolodner and Tewari, 1972, 1975 Hermann et al 1975). Although the length of ct DNA is variable between different genera, it is fixed within a single species. The molecular weight of ct DNA falls in the range 85.2×10^6 - 143×10^6 (Meyer and Herrman,1973; Kolodner and Tewari, 1975a; Behn and Herrman, 1977). Evidence from denaturation and restriction mapping indicates that the circles present within any one species have the same sequence (Kolodner and Tewari, 1975b; Bedbrook and Bogorad, 1976; Whitfeld et al 1978).

Reassociation Kinetic studies of ct DNA species show that all ct DNAs contain a single component of kinetic complexity equal to a molecular weight of 1×10^8 (Kolodner and Tewari, 1975a) with the exception of Acetabularia which has ct DNA of complexity 1.1×10^9. However, there is some evidence that ct DNA may contain repeated sequences of two types. Renaturation (Kolodner and Tewari, 1979) and restriction studies of maize (Bedbrook and Bogorad, 1976) lettuce (Hobom et al, 1977) Chlamydomonas (Rochaix, 1978) and spinach (Whitfeld et al, 1978) ct DNAs show that the DNA sequence contains a large region which is repeated in reverse orientation. Electron micrographs show that the repeated sequence is between 22500 and 24000 base pairs in length and corresponds to about 16% of the DNA circle. The inverted repeat sequence is not a universal feature of ct DNA: pea and Euglena gracilis genomes have no such structure

(Gray and Hallick 1977, 1978; Kolodner and Tewari, 1979).

The second type of repeated sequence is small (about 100-300 base pairs in length) and is distributed throughout the genome of Chlamydomonas reinhardii (Gelvin and Howell, 1979; Rochaix, 1978) and maize (Bedbrook and Bogorad, 1975). Ribonucleotides are also present at 18 sites in pea and spinach DNA and 12 sites in lettuce DNA (Kolodner et al, 1975, 1976). The function of these ribonucleotides is unknown.

A chloroplast DNA circle of 45 μm contour length has a coding capacity of about 6×10^6 daltons of polypeptide, assuming asymmetric transcription and allowing for an inverted repeat sequence (Highfield and Ellis, 1978). The investigation of the polypeptides encoded within this genome has used three major approaches: genetic crossing, hybridization and direct mapping.

The property of Nicotiana species to form interspecific hybrids has been used extensively by Wildman and co-workers to investigate the site of coding of some chloroplast proteins. Genes which are present on ct DNA are only transmitted by the maternal line (via the egg) whereas nuclear genes obey the rules of classical Mendelian genetics (Wildman et al, 1975). If a protein from two parent species can be distinguished, it is possible to determine if the gene is inherited maternally, or via both parents. Characteristics of the subunits of Fraction I protein which have been used in such experiments are tryptic peptide patterns (Chan and Wildman, 1972; Kawashima and Wildman, 1972), isoelectric focusing variants (Sakano et al, 1974) amino acid composition (Kung, 1977; Kawashima et al, 1971; Kung et al, 1974) and enzymic activity (Singh and Wildman, 1973). The data from such experiments indicate that the large subunit of Fraction I protein is encoded in ct DNA, whilst the small subunit is encoded in the nuclear genome. Maternal inheritance

of the biochemical properties of Fraction I protein have also been recorded in wheat (Chen et al, 1975) and oat (Steer, 1975).

The genetic mapping technique has been extended successfully to map chloroplast proteins other than Fraction I protein. Using the isoelectric points of ferredoxin polypeptides from different Nicotiana species, Wildman et al (1975) have shown that the genetic information for ferredoxin resides in the nuclear genome. Similarly, tryptic peptide mapping of the chlorophyll a/b binding proteins from Nicotiana interspecific hybrids indicates that this polypeptide is also encoded in the nuclear genome (Kung et al, 1972). However, this technique has proved less successful with a multisubunit complex the CF_1 protein. Kwanyuen and Wildman (1978), using isoelectric focusing of CF_1 subunits from Nicotiana species were only able to demonstrate that two subunits are encoded in ct DNA and one in nuclear DNA. The identity of these subunits could not be resolved. In most cases, genetic mapping has proved a useful tool for the location of genes coding for chloroplast polypeptides. It is, however, open to the criticism that the technique may be following modifying genes rather than the structural genes themselves (Bedbrook and Kolodner, 1979), and that the results of the crosses have not been analysed further by genetic methods (Kung, 1977).

The unicellular green alga Chlamydomonas reinhardii has also been used to study chloroplast genetics. This subject has recently been reviewed by Gillham et al (1978). Sexual reproduction in Chlamydomonas involves the fusion of two gametes, each of which contains a chloroplast. When the gametes fuse, the chloroplasts also fuse (Cavalier-Smith, 1970). However, only the maternal copies of the chloroplast genome survive in the zygote enabling the locating of genes in ct DNA by identifying traits which show maternal inheritance. The paternal ct DNA genes may be removed by a restriction/modification system after gamete fusion (Sager and Ramanis, 1974.

Using this method, it has been shown that some genes affecting chloroplast ribosomes are inherited uniparentally (Mets and Bogorad, 1972; Boynton et al, 1972) whilst others are inherited in a Mendelian fashion (Burton, 1972; Schlanger et al, 1972). Such mutations affect changes either in ribosomal proteins (Mets and Bogorad, 1972; Davidson et al, 1974) or in ribosome assembly (Harris et al, 1974).

The inheritance of the major polypeptide components of CPI and CPII have been examined in Chlamydomonas and Nicotiana. (Gillham et al, 1978). Kung et al (1972) followed the inheritance of CPII polypeptides by tryptic peptide mapping. Nicotiana tabacum and N. glauca CPII chlorophyllins each contain a unique tryptic peptide, designated T and G respectively. In reciprocal interspecific hybrids, the T peptide was inherited in a Mendelian fashion, implying that the CPII polypeptide containing fragment T is encoded in a nuclear gene.

In Chlamydomonas, the inheritance of CPI, the chlorophyllin associated with Photosystem I activity (Section I2A), has been examined using CPI and photosystem I-deficient mutants. Chua et al (1975) were able to show that Mendelian mutants F1 and F14 which lack CPI also lack a thylakoid polypeptide (designated 2) which was the sole CPI polypeptide in the wild-type. Bennoun et al (1977) have also described a maternally-inherited mutation of CPI. The mutant thylakoids contain 10-15% of wild type amounts of CPI, and a similarly reduced amount of polypeptide 2. It appears, therefore, as if both chloroplast and nuclear genes are involved in the synthesis of CPI in Chlamydomonas.

Maternally-inherited mutations of Antirrhinum have proved a useful tool for genetic studies. The plastome mutant en: alba -1,

which lacks CPI, also lacks a polypeptide which may be functionally equivalent to the CPI polypeptide (Herrmann 1971). The problem with using such Chlamydomonas and Antirrhinum mutants to follow the inheritance of chlorophyllins is that many of the mutations are pleiotropic. For example, Chua and Bennoun (1975) have described two Mendelian mutations, F34 and T4 (a temperature-sensitive mutation) which may directly or indirectly affect the synthesis of polypeptides 5 and 6. The complexity of such an approach has led to the development of other techniques to examine the genes encoded in ct DNA. The most successful of these is direct gene mapping.

The approach adopted in direct gene mapping is to cleave the ct DNA being studied into a number of specific fragments using restriction endonucleases. The fragments from each enzyme employed can be used to generate a physical restriction map. Such maps have been generated for Eugena gracilis (Gray and Hallick, 1977), Chlamydomonas reinhardii (Rochaix, 1977, 1979), maize (Bedbrook and Bogorad, 1976) and spinach (Hobom et al, 1977; Crouse et al, 1978; Whitfeld et al 1978). Genes on restriction fragments can be located by DNA : RNA hybridization with known RNA molecules or by coupled transcription-translation of the fragments, and identification of the resultant polypeptides on gels.

The hybridization approach has been used to map chloroplast rRNA molecules onto restriction maps. Hybridization of purified rRNA from chloroplast ribosomes to total ct DNA had revealed that each ct DNA circle contains sequences complementary to two copies of 16S and 23S ribosomal RNA (rRNA) per genome in pea, spinach, oat, bean and maize (Tewari and Wildman, 1968; Thomas and Tewari, 1974). By hybridization of rRNA to restriction fragments and electron micro-

scopic visualization, these data have been confirmed, and the 16S and 23S rRNA molecules mapped inside the inverted repeat sequences of maize (Bedbrook et al, 1977), spinach (Whitfeld et al, 1976) and Chlamydomonas (Rochaix and Malnoe, 1978a). These data are summarized in Table B. In the aforementioned species, the rRNA gene order in each repeat is similar to that observed in E.coli DNA i.e. 16S-23S-5S (Lund et al, 1976).

In the inverted repeat regions, the 16S and 23S rRNA genes are separated by a spacer region of 1680-2200 base pairs in length, depending on the organism studied (Bedbrook et al, 1977; Whitfeld et al, 1978; Rochaix and Malnoe, 1978a). In Chlamydomonas, 3S and 7S RNA species are transcribed from this spacer region; their function is unknown (Rochaix and Malnoe, 1978).

Chloroplast transfer RNA (tRNA) species have been located on the chloroplast genome by hybridization (Haff and Bogorad, 1976; Tewari et al, 1977). However, unlike rRNA genes, the tRNA genes mapped onto spinach, Euglena and Chlamydomonas ct DNAs are scattered throughout the genome (Table B).

Recent evidence suggests that in Chlamydomonas ct DNA, the 23S rRNA gene is interrupted by a 940 base pair intervening sequence, 270 base pairs from the 5' end of the coding strand (Rochaix and Malnoe, 1978a). Such a sequence has not been detected in maize or spinach chloroplast rRNA genes. Intervening sequences have previously been detected in eukaryotic DNA in the rabbit β globin gene (Jeffreys and Flavell, 1977), mouse λ light chain gene (Brack and Tonegawa, 1977) the ovalbumin gene (Breathnach et al, 1977) as well as Drosophila rRNA (Glover and Hogness, 1977) and yeast tRNA genes (Goodman et al, 1977). The function of these inserts is not yet known. The numerous small repeat sequences detected by Gelvin

RNA species	Organism	No. of copies per circle	Location on circle	Reference
16S rRNA	*Zea mays*	2	In inverted repeats	[1]
	Spinacia oleracea	2	In inverted repeats	[2]
	Chlamydomonas reinhardii	2	In inverted repeats	[3]
	Euglena gracilis	4	Three in tandem unit, one separate	[4,5,6]
23S rRNA	*Zea mays*	2	In inverted repeats	[1]
	Spinacia oleracea	2	In inverted repeats	[2]
	Chlamydomonas reinhardii	2	In inverted repeats	[3]
	Euglena gracilis	3	In tandem repeats	[4,5]
4.5S rRNA	*Spinacia oleracea*	2-4	In inverted repeats between 23S rRNA and spacer	[2,7]
tRNAs	*Spinacia oleracea*	-	In inverted repeat regions and either side	[8]
	Euglena gracilis	-	Distributed over whole circle	[5,6]
	Chlamydomonas reinhardii	-	One species maps between 16S and 23S rRNAs. Remainder are distributed over whole circle	[3,9]

Table B RNA genes mapped onto chloroplast DNA

[1] Bedbrook et al (1977)

[2] Whitfeld et al (1978)

[3] Rochaix and Malnoe (1978a)

[4] Jenni and Stutz (1979a)

[5] Hallick et al (1978)

[6] Jenni and Stutz (1978b)

[7] Hartley (1979)

[8] Bohnert et al (1979)

[9] Malnoe and Rochaix (1979)

and Howell (1979) may prove to be intervening sequences when mapped.

Only two genes coding for chloroplast proteins have been mapped onto ct DNA (Table C). The first of these, the LSU gene, has been mapped by *in vitro* transcription and translation of cloned restriction fragments. In maize, the LSU gene occupies a 2500 base pair fragment 30 000 base pairs from the 5' end of the closest set of rRNA genes (Coen *et al*, 1977). Since the minimum coding sequence length for LSU is 1500 base pairs, it is probably present at one copy per DNA circle. In *Chlamydomonas*, there is some contradiction between data from two different studies. Howell *et al* (1977) and Gelvin *et al* (1977) have used a partially purified LSU mRNA to identify cloned restriction fragments of ct DNA. Their data indicate that LSU RNA hybridizes to the inverted repeat region, and they have suggested that the transcription of LSU and rRNA genes is linked. However, there is a strong possibility that their LSU mRNA hybridization probe was contaminated with other RNA species. Rochaix and Malnoe (1978b) find that the LSU gene maps outside the inverted repeat sequence and is present at one copy per DNA circle.

A thylakoid polypeptide of 32000 molecular weight and unknown function has been described in pea (Eaglesham and Ellis, 1974), spinach (Bottomley *et al*, 1974) and maize (Grebanier *et al*, 1978). It is not known whether the polypeptide is of the same structure and function in these three species. In maize, however, the gene for such a polypeptide has been located on the chloroplast genome (Bedbrook *et al*, 1978). No data is as yet available for other species on this point.

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Gene	Species	Number of copies per circle	Location on circle	Reference
LSU	*Zea mays*	1	In 1500 base pair sequence outside the inverted repeat	[1]
LSU	*Spinacia oleracea*	-	-	[2]
LSU	*Chlamydomonas reinhardii*	1	In 5500 base pair EcoRI fragment outside the inverted repeat	[3]
LSU	*Chlamydomonas reinhardii*	-	Inside the inverted repeat	[4,5]
32 000 m.wt. thylakoid polypeptide	*Zea mays*	-	outside inverted repeat	[6]

Table C Polypeptide genes mapped onto chloroplast DNA

[1] Bedbrook et al (1979)

[2] Bottomley and Whitfeld (1978)

[3] Rochaix and Malnoe (1978b)

[4] Howell et al (1977)

[5] Howell and Gelvin (1978)

[6] Bedbrook et al (1978)

I2C. The sites of synthesis of chloroplast proteins.

Chloroplasts contain a protein synthetic machinery based on 70S ribosomes which is quite distinct from the 80S-based cytoplasmic machinery (Ellis, Blair and Hartley, 1973). Isolated chloroplast ribosomes have many common properties with bacterial ribosomes including their sedimentation coefficients (Stutz and Noll, 1967) and RNA composition (Loening and Ingle, 1967; Payne and Dyer, 1971).

Chloroplast ribosomes also share functional similarities with bacterial ribosomes which makes them differ from the cytoplasmic, eukaryotic ribosomes. Initiation of protein synthesis on chloroplast ribosomes requires f-met $tRNA_f$ (Schwartz et al, 1967), a feature of bacterial protein synthesis. In view of these structural and functional homologies, it comes as no surprise that chloroplasts are also sensitive to inhibitors of protein synthesis by bacterial 70S ribosomes, but insensitive to inhibitors of 80S ribosomes (Boulter et al, 1972; Ellis, 1969, 1970). This differential sensitivity has been used as a means of determining which polypeptides are synthesized on chloroplast ribosomes and which polypeptides are synthesized on cytoplasmic ribosomes.

The interpretation of inhibitor data is dependent upon the recognition of certain points concerning the operation of the inhibitor chosen (Ellis et al, 1973; Ellis, 1976). Many inhibitors affect functions other than protein synthesis (Ellis and Macdonald, 1970; Wara-Aswapati and Bradbeer, 1974). All four stereoisomers of chloramphenicol affect uptake and oxidative phosphorylation, but only D-threo chloramphenicol is specific for protein synthesis on chloroplast ribosomes (Ellis, 1969). At high concentrations (300 µg/ml), D-threo and L-threo chloramphenicol inhibit phosphorylation and phosphorylating electron transport in isolated spinach

chloroplasts (Wara-Aswapati and Bradbeer, 1974).

Despite the problems of using inhibitors, this approach has yielded some useful information which helped the determination of which polypeptides are synthesized inside the chloroplast. The results of this approach have been summarized by Ellis et al (1973), and more recently by Kirk and Tilney-Bassett (1978). From the assembled data, it is clear that, with the exception of Fraction I protein, the Calvin cycle enzymes are synthesized on 80S ribosomes. Fraction I protein is the product of both chloroplast and cytoplasmic protein synthesis; inhibitor data from etiolated plants which are greened in the presence of isotope suggest that LSU is synthesized on 70S ribosomes whilst SSU is synthesized on 80S ribosomes (Ellis and Hartley, 1971; Ireland and Bradbeer, 1971; Criddle et al, 1970). Ferredoxin and Ferredoxin-NADP reductase are the products of cytoplasmic protein synthesis (Armstrong et al, 1971; Haslett et al, 1973). The chloroplast coupling factor requires both chloroplast and cytoplasmic protein synthesis (Horak and Hill, 1971, 1972); the α,β and ϵ subunits are synthesized on 70S ribosomes whilst the γ and δ subunits are synthesized on 80S ribosomes (Bouthyette and Jagendorf, 1978). The synthesis of ribosomal proteins also appears to require both 70S and 80S ribosomes (Ellis and Hartley, 1971; Margulies, 1971; Freyssinet, 1977).

A significant advance in the study of the sites of synthesis of chloroplast polypeptides was the successful isolation of intact chloroplasts capable of protein synthesis in vitro (Blair and Ellis, 1973). Many earlier attempts to achieve this failed because of bacterial contamination and low rates of incorporation (App and Jagendorf, 1963; Spencer and Wildman, 1964; Goffeau and Brachet, 1965; Spencer, 1965; Hall and Cocking, 1966; Davies and Cocking,

1967; Gnanam et al, 1969; Margulies, 1970). These problems were circumvented by Blair and Ellis by using chloroplasts capable of high rates of photophosphorylation (Ramirez et al, 1968). These plastids carry out protein synthesis which is RNAase-resistant and light dependent, characteristics expected of protein synthesis in intact chloroplasts.

The results of this approach are summarized in Table D. This table is intended to list only those polypeptides which have an identified function. The exception is the 32000 molecular weight thylakoid protein. A polypeptide in this size range is synthesized in chloroplasts isolated from maize, spinach, pea and Euglena (Table D). In all these systems it is heavily labelled, yet does not correspond to a major stained band. Since no function has, as yet, been ascribed to this polypeptide, it is not possible to say whether the 32000 molecular weight protein is identical in these organisms. Such a polypeptide was first described by Eaglesham and Ellis (1974), and given the name peak D from its mobility on polyacrylamide gels. For brevity, in this thesis the polypeptide of the same mobility and properties described above will be called peak D in spinach. No further similarity is implied by this name.

Table D emphasizes the relatively small number of chloroplast protein-synthetic products which have been identified. Two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) of soluble polypeptides synthesized in isolated pea chloroplasts reveals about 80 discrete products (Ellis et al, 1978). SDS polyacrylamide gradient slab gels of thylakoid polypeptides show that at least 25 of these polypeptides are synthesized in isolated chloroplasts (Ellis, 1977). The function of the majority of these polypeptides is unknown.

At the present time, there is no isolated chloroplast system available in which transcription and translation are coupled. Protein

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Polypeptide	Species	Reference
LSU	Pisum sativum	[1]
	Spinacia oleracea	[2,3]
	Euglena gracilis	[4]
32000 m.wt.	Pisum sativum	[5]
thylakoid	Zea mays	[6]
polypeptide	Spinacia oleracea	[2,3]
α,β and ϵ	Pisum sativum	[7]
CF_1 subunits	Spinacia oleracea	[8]
Elongation factors	Spinacia oleracea	[9,10]
Cytochrome f	Pisum sativum	[11]
	Spinacia oleracea	[12]
Cytochrome b_{-558}	Spinacia oleracea	[12]
DCCD-binding protein	Pisum sativum	[13]
PSI apoprotein	Spinacia oleracea	[14]
	Acetabularia mediterrania	[15]

Table D Polypeptides synthesized by isolated chloroplasts.

[1] Blair and Ellis (1973)

[2] Bottomley et al (1974)

[3] Morgenthaler and Mendiola-Morgenthaler (1976)

[4] Vasconcelos (1976)

[5] Eaglesham and Ellis (1974)

[6] Grebanier et al (1978)

[7] Ellis (1977)

[8] Mendiola-Morgenthaler et al (1976)

[9] Tiboni et al (1976)

[10] Cifferi and Tiboni (1976)

[11] Docherty and Gray (1979a)

[12] Zielinski and Price (1978)

[13] Docherty and Gray (1979b)

[14] Zielinki and Price (in press)

[15] Green (in press).

synthesis in intact pea chloroplasts is insensitive to Actinomycin D, an inhibitor of RNA synthesis (Blair and Ellis, 1973). Translation is therefore dependent on mRNA synthesized before isolation. However, Highfield and Ellis (1976) have shown that pea chloroplasts reinitiate protein synthesis on this mRNA several times after isolation. It is not known whether the relative amounts of each polypeptide synthesized is changed on isolation.

Other cell-free systems have been used to study the sites of synthesis of chloroplast polypeptides. One approach is to isolate polysomes and discharge the polypeptides being synthesized with [^{3}H] puromycin. The polypeptides may then be identified by means of specific antibodies. This approach has been used successfully by Gooding et al (1973) to show that wheat 70S polysomes synthesize LSU whilst 80S polysomes synthesize SSU. If total leaf polysomes are allowed to "run off" in a cell-free system from wheat germ in the presence of a radioactive amino acid, the polypeptides being synthesized are labelled. Using specific antibodies, it is possible to show that SSU is synthesized on 80S ribosomes in a "run-off" system (Roy et al, 1977, Gray and Kekwick, 1974). The lack of incorporation of isotope into LSU in these systems, despite the presence of 70S polysomes, has been interpreted as a reflection of the cell-free system employed for 80S ribosomes.

The more direct approach of translation of isolated chloroplast and cytoplasmic mRNA in a cell-free system has been employed to determine sites of synthesis of chloroplast polypeptides. The cytoplasmic site of synthesis has been inferred for polypeptides if their mRNA is contained in the polyadenylated mRNA fraction in leaf RNA. Chloroplast mRNA contains little or no poly(A) (Wheeler and Hartley, 1975; Sano et al, 1979) whereas cytoplasmic mRNA is polyadenylated to varying degrees (Cashmore et al, 1976; Howell and

Gelvin, 1978). Poly(A)-containing RNA has been translated in a wheat germ extract to yield polypeptides having immunological identity with SSU (Cashmore et al, 1978; Highfield and Ellis, 1978; Dobberstein et al, 1977), the chlorophyll a/b binding protein (Apel and Klopstech, 1978) and ferredoxin (Huisman et al, 1978). However, these polypeptides are synthesized as larger precursors which, in the case of SSU, has been shown to be taken up by isolated chloroplasts and cleaved to the mature polypeptide (Highfield and Ellis, 1978; Smith and Ellis, 1979; Chua and Schmidt, 1978).

D. Conclusions.

The aim of this section has been to review the structure of the chloroplast in relation to its biochemical constituents. Although chloroplasts contain DNA and a protein-synthetic machinery, they are only able to synthesize a limited spectrum of polypeptides. A small number of these genes has been mapped directly onto chloroplast DNA; the site of encodement of the remainder is inferred from studies with inhibitors or isolated chloroplasts. Implicit in this inference is the assumption that nucleic acids do not cross the chloroplast envelope; that is, that mRNA transcribed from nuclear genes does not cross the chloroplast envelope and undergo translation on 70S ribosomes. There have been recent claims that poly(A) RNA crosses the chloroplast envelope (Verdier, 1978a and b) but cytoplasmic contamination has not been discounted. It has also been claimed that tRNA synthesized in the chloroplast enters the cytoplasm in Euglena (McCrea and Hershberger, 1978) and counterclaimed (Schwartzbach et al, 1978). To date there is no good evidence that mRNA transcribed from nuclear genes is translated inside chloroplasts.

I3. CHLOROPLAST DEVELOPMENT FROM ETIOPLASTS AND PROPLASTIDS

A. Proplastids and etioplasts.

The aim of this section is to describe two forms of plastid, proplastids and etioplasts, and their route of development into the mature chloroplast described in Section I2. The review will concentrate on Angiosperm development.

A proplastid has been defined as a "small, colourless or pale green, undifferentiated plastid occurring in the meristematic cells of shoot and root" (Kirk and Tilney-Bassett, 1978). Proplastids are spherical or ellipsoid in shape and may be amoeboid, with a diameter of between 0.4 and 0.9 µm (Muhlthaler, 1971; Kirk and Tilney-Bassett, 1978). The structure of this organelle has been studied by von Wettstein and Kahn (1960). Electron micrographs reveal that they consist of a double membrane-bounded sac containing a more or less homogeneous matrix. This matrix contains occasional vesicles and some thylakoid stacks (Menke, 1960). Irregularly-shaped clear areas are often evident in the matrix, and the fibrillar material observed in these has been suggested to be DNA (Whatley et al, 1972). The internal membrane sometimes invaginates into the matrix in the form of tubes, and these membranes are sometimes associated with a starch granule (Kirk and Tilney-Bassett 1978; Bradbeer et al, 1974a).

When higher plants and green photosynthetic algae are grown in the dark, the proplastids differentiate into a form termed the etioplast (Kirk and Tilney-Bassett, 1978). This plastid type lacks chlorophyll and the thylakoid membrane structure of chloroplasts described in Section IA. Etioplasts are ellipsoid and have a diameter of 1-3 µm (Mego and Jagendorf, 1961). The etioplast is bounded by a double envelope (Rebeiz et al, 1973); the interior

contains stroma filled with ribosomes. The DNA is visible in areas of low electron density (Jacobson, 1968; Gyldenholm, 1968; Sprey and Geitz, 1973). In some oat etioplasts there are large crystalline aggregates of Fraction I protein termed stromacentres. (Gunning et al, 1968).

Within the stroma, the most obvious lamellar system is the prolamellar body (Leyon, 1954a,b; Heitz, 1954). Each etioplast contains up to four of these arrays of interconnected tubules (Gunning, 1965). Around the edges of the prolamellar body, there are sometimes visible a few double-membrane lamellae which have been termed thylakoids (Kirk and Tilney-Bassett, 1978). The three-dimensional structure of the prolammellar body has been studied by electron microscopy (Wehrmeyer, 1965a,b,c; Ikeda, 1968; Weier and Brown, 1970). In oat, the lattice is formed of basic tetrahedral units comprising four tabules (Gunning and Steer, 1975). The resultant hexagonal lattice is continuous inside with the stroma. Other formations have been found in Phaseolus, where a cubic arrangement is visible (Gunning, 1965; Gunning and Jagoe, 1967; Granick, 1961).

The internal structure of the etioplast thylakoids has been investigated using electron microscopy. The etioplast thylakoids have surface particles of 10 nm diameter which may represent CF_1 particles (Kahn, 1968). In addition, freeze-fracture studies of barley etioplast thylakoids show that one fracture face contains two size-classes of particle of 6-12 nm and 9-12 nm diameters (Phung Nhu Hung et al, 1970). These particles, on the basis of size, numbers and distribution, may represent photosystems I and II precursor particles.

The protein and lipid content of etioplasts have been analyzed.

Isolated maize etioplasts contain monogalactosyl and digalactosyl diglycerides, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl inositol and phosphatidyl ethanolamine as the major lipids (Tevini, 1972; Mackender, 1978). The relative amounts of these lipids change as etioplasts develop. The acyl lipid composition of etioplasts is qualitatively similar to that found in mature chloroplasts, except that etioplasts contain very little trans Δ^3 hexadecanoic acid (Leech et al, 1973; Leese and Leech, 1976).

The etioplast envelope of Avena contains 16 major polypeptides ranging in molecular weight from 25000 to 117000 (Cobb and Wellburn, 1974). The identities of these polypeptides is unknown. In addition to these polypeptides, bean etioplasts contain phytochrome in their envelopes as judged by spectrophotometry (Evans and Smith, 1976a,b). These authors speculate that phytochrome may act as a permease, regulating metabolite movement in and out of the organelle during development. It is known that the etioplast envelope is highly permeable to small molecules (Hampp and Schmidt, 1976; Cockburn and Wellburn, 1972).

Avena prolamellar bodies contain seven major SDS-soluble polypeptides ranging in molecular weight from 22000 to 68000 (Lütz, 1975). Of these, the 22000 molecular weight polypeptide was found to be a glycoprotein also present in the thylakoids of mature Avena chloroplasts. A similar analysis of prolamellar bodies from maize has also revealed the presence of seven SDS-soluble polypeptides, ranging from 25000 to 102000 in molecular weight (Forger and Bogorad, 1973). Using a more sensitive gel system, Grebanier et al (1979) have shown that maize prolamellar bodies contain many more polypeptides than the earlier study suggested. They conclude that there are three classes of polypeptide present in the plastid inner membranes:-

1. polypeptides present in the prolamellar body which disappear on greening;
2. polypeptides absent in the etioplast which appear during greening; and
3. polypeptides present in both etioplasts and chloroplasts.

Thus it is clear from these studies that etioplast inner membranes do not contain the same spectrum of polypeptides found in thylakoids. A similar conclusion was reached by Nielson (1975) using barley seedlings.

The identity of these classes of polypeptide has only been shown in a few cases. In the first class of plastid polypeptide, it is clear that bean and maize etioplasts contain CF_1 activity (Lockshin et al, 1971; Gregory and Bradbeer, 1975; Horak and Hill, 1971) in amounts comparable to that detected in mature chloroplasts. Such CF1 particles can be used to restore coupling factor activity to stripped chloroplast membranes (Bogorad, 1975). In addition, bean etioplasts contain many of the photosynthetic enzymes present in chloroplast, but in reduced amounts (Bradbeer et al, 1974a). Barley and bean etioplasts contain cytochrome f , b_{563} and b_{559LP}, as well as plastocyanin (Plesnicar and Bendall, 1972; Whatley et al, 1972; Phung Nhu Hung et al, 1972; Gregory and Bradbeer, 1973). These components are also found in amounts comparable to those in mature chloroplasts. So far, the identity of the etioplast-specific polypeptides is unknown.

Isolated etioplasts contain the components of a protein-synthetic machinery, namely ribosomes (Boardman, 1966; Dyer et al, 1971) tRNA and amino acid activating enzymes (Guillemaut et al, 1972; Burkhard et al, 1972). On isolation and incubation in vitro with an added energy source, pea etioplasts synthesize the large subunit of Fraction I protein as a major product, in addition to at

least 5 unidentified polypeptides (Siddell and Ellis, 1975).

B. Chloroplast development during greening.

The lack of biochemical characterization of proplastids is largely due to the difficulty of isolating and identifying preparations in suitable quantities. The majority of studies of chloroplast development have therefore used etioplasts as the starting point, rather than proplastids. Kirk and Tilney-Bassett (1978) state that, "The etioplast is best regarded as a developmental stage in the formation of a chloroplast". In fact, the etioplast is the furthest stage that a plastid can achieve in the absence of light.

During the developmental transition of etioplast to chloroplast induced by light, there are marked ultrastructural and biochemical changes. The most obvious of these is the conversion of the prolamellar body from a tight, well-defined crystalline mass into perforated lamellar sheets and finally into the array of grana and thylakoids of the mature chloroplast. This process has been relatively well-described at the ultrastructural level from the electron microscopy of thin sections (Von Wettstein, 1958; Gyldenholm, 1968; Weier et al, 1970; Weier and Brown, 1970; Gunning and Jagoe, 1967) in a number of plant species.

On illumination of an etiolated plant, there is a rapid photoconversion of protochlorophyll to chlorophyll. Under the electron microscope, the prolamellar body can be seen to lose crystallinity. The process of thylakoid formation has been quantitated with respect to membrane reorganization and synthesis (Bradbeer, 1975; Henningson and Boynton, 1970; Weier et al, 1970; Robertson and Laetsch, 1974). In bean, the conversion of the prolamellar body into porous membrane sheets accounts for all the membrane formation during the first few hours of greening under continuous light (Bradbeer 1970, 1975).

After this stage, there is de novo synthesis of thylakoids and formation of grana. A similar time-course has been observed by Henningson and Boynton (1970) in barley. In younger etioplasts, the loss of crystallinity in the prolamellar body is simultaneous with the initial rapid protochlorophyll reduction (Henningson and Boynton, 1970); in older plastids there may be a lag in prolamellar body breakdown.

When new thylakoid material is synthesized, there also begins the stage of grana formation. Robertson and Laetsch (1974) have quantitated this process in greening barley leaves. The advantage of the barley system is that the plastids form a developmental gradient of increasing age towards the tip of the leaf. In this system, it is clear that younger etioplasts take longer to form mature chloroplasts than do older etioplasts. During the period of grana formation, there is a massive increase in total thylakoid area in bean (Bradbeer et al, 1974b). The new thylakoid material is formed by de novo synthesis; the lipid being formed in the inner envelope; the inner envelope membrane contains the enzymes of the final stages of galactolipid synthesis (Douce, 1974; Douce and Joyard, 1978).

During the period of grana formation, there is increased chlorophyll and protein synthesis. However, there is not always a lag phase between the initial rapid reduction of protochlorophyll and de novo chlorophyll synthesis. Bean seedlings show a lag phase in chlorophyll synthesis which is related to the length of etiolation; young seedlings have no lag phase (Sisler and Klein, 1963; Wolff and Price, 1960). The course of chlorophyll synthesis during greening has been reviewed by Kirk (1970) , and Treffry(1978).

During greening there is an increase in the rate of protein and RNA synthesis (Ingle, 1968; Smith et al, 1970; Dyer et al, 1971;

Brantner and Dure, 1975). Etioplasts assayed *in vitro* have a smaller protein synthetic capacity than chloroplasts isolated from plants of the same age grown under light conditions (Drumm and Margulies, 1970; Hearing, 1973). Illumination of etiolated maize seedings brings about an increase in plastid DNA-dependent RNA polymerase activity (Apel and Bogorad, 1976) and increased RNA synthesis (Harel and Bogorad, 1973). In maize, there is an increased synthesis of the mRNA for the 32000 molecular weight thylakoid polypeptide precursor, as judged by hybridization and translation studies (Coen *et al*, 1978). A similar increase in translatable mRNA for the 32000 molecular weight polypeptide precursor has been observed in greening *Spirodela* (Edelman and Reisfeld, 1978). Cytoplasmically-synthesized chloroplast polypeptides are also induced by light. In *Lemna* and barley, precursor polypeptides to the small subunit of Fraction I protein and the chlorophyll *a/b* binding protein are the major products of *in vitro* translation of mRNA from greening tissue (Tobin, 1978; Apel, 1979).

Although etioplasts contain substantial amounts of the Calvin cycle enzymes, including Fraction I protein, there is an increased synthesis of these polypeptides during greening (Ireland and Bradbeer, 1975; Chen *et al*, 1967). There is an increase in ribulose bisphosphate carboxylase activity in greening barley (Kleinkopf *et al*, 1970; Smith *et al*, 1974; Keller and Huffaker, 1967), maize (Chen *et al*, 1967), pea (Graham *et al*, 1968)&bean (Gray and Kekwick, 1974; Ireland and Bradbeer, 1971). The increase in ribulose bisphosphate carboxylase activity is probably due to *de novo* synthesis of the enzyme rather than to light activation (Sakano and Wildman, 1974; Bassham, 1971) or to delayed degradation because the newly-synthesized polypeptides can be immunoprecipitated from polysomes (Roy *et al*, 1975; Alscher *et al*, 1976), and the increase in activity

Brantner and Dure, 1975). Etioplasts assayed *in vitro* have a smaller protein synthetic capacity than chloroplasts isolated from plants of the same age grown under light conditions (Drumm and Margulies, 1970; Hearing, 1973). Illumination of etiolated maize seedings brings about an increase in plastid DNA-dependent RNA polymerase activity (Apel and Bogorad, 1976) and increased RNA synthesis (Harel and Bogorad, 1973). In maize, there is an increased synthesis of the mRNA for the 32000 molecular weight thylakoid polypeptide precursor, as judged by hybridization and translation studies (Coen *et al*, 1978). A similar increase in translatable mRNA for the 32000 molecular weight polypeptide precursor has been observed in greening *Spirodela* (Edelman and Reisfeld, 1978). Cytoplasmically-synthesized chloroplast polypeptides are also induced by light. In *Lemna* and barley, precursor polypeptides to the small subunit of Fraction I protein and the chlorophyll *a/b* binding protein are the major products of *in vitro* translation of mRNA from greening tissue (Tobin, 1978; Apel, 1979).

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is prevented by inhibitors of protein synthesis (Gregory and Bradbeer, 1975; Keller and Huffaker, 1967). A similar situation has been observed with respect to CF_1 synthesis in bean and maize (Horak and Hill, 1971; Lockshin et al, 1971).

Attempts have been made to correlate the onset of photosynthetic competence by developing chloroplasts with changes in plastid morphology. Hiller et al (1977) using greening pea and barley have shown that PSI activity can be detected before CPI formation, as judged by analysis of chloroplast membranes by SDS polyacrylamide gel electrophoresis. In barley, electron transport dependent on photosystem I activity can be detected within a few minutes after the commencement of illumination, but declines relative to chlorophyll during greening (Plesnicar and Bendall, 1973). No p700 was detected at this stage. Photosystem II activity and grana formation were not detected until at least 2½ hours after the commencement of illumination. These data differ from those of Alberte et al 1977) who correlated an increase in photosystem I activity with CPI formation in jackbean. However, this study was carried out at high relative humidity, which abolishes the lag phase of chlorophyll synthesis. The most likely explanation of these observations is that the small amount of protochlorophyll present in etioplasts is rapidly converted to chlorophyll on illumination, and this has some activity in photosystem I. However, formation of CPI and CPII is dependent on the latter, de novo synthesis of chlorophyll a and b.

Intermittent illumination rather than continuous illumination has been used to correlate photosynthetic activity and grana formation. Intermittently illuminated bean leaves, which have been illuminated for only 2 minutes in every 100 minutes, form only CPI; CPII is completely absent (Argyroudi-Akoyunoglou et al, 1971). A

similar observation has been made in pea, and has been shown to include a lack of CPII apoprotein, the chlorophyll a/b binding protein (Armond et al, 1977). Such plants do not contain grana, but only single thylakoids (Greef et al, 1971; Bradbeer et al, 1970). Maize illuminated for 4 seconds in every 4 hours lacks CPI and CPII yet has photosystem I and II activities (Acker et al, 1976). However, some kind of complex was present in these leaves, consisting of chlorophyll and 21000 and 29000 molecular weight polypeptides. Such plants can develop CPI and CPII when transferred to continuous light, and there is an increase in particle size on the inner freeze-fracture face of the developing thylakoids (Davis et al, 1976; Armond et al, 1976; Armond et al, 1977). Armond et al (1977) attribute these structural changes to the addition of CPII light harvesting polypeptides to the photosystem complex already present.

A number of studies have been concerned with the control of protein and chlorophyll synthesis during greening. The involvement of phytochrome in chloroplast development has recently been reviewed by Mohr (1977).

I3C. Chloroplast development under a duirnal light regime.

Chloroplast development from proplastids is relatively less well characterized than is chloroplast development from etioplasts. Much of the early stages of this developmental sequence have been studied only by electron microsopy, because of the difficulty of isolating proplastids.

Meristematic cells contain colourless, amoeboid proplastids, containing relatively little internal structure (Section I3A). The development of these plastids into mature chloroplasts begins with the differentiation of the meristem into mesophyll and palisade leaf cells. This sequence has been described by Buvat (1958),

Muhlthaler and Frey-Wyssling (1959) and von Wettstein (1958), and may be summarised as involving an increase in plastid volume with a concommitant increase in thylakoid area. The thylakoids appear to form from the inner envelope membran e as has been described during the later stages of greening.

During leaf expansion there is increased chlorophyll synthesis in pea (Smillie and Krotkov, 1961) and bean (Dale and Murray, 1969). In wheat, where young leaves are shaded by the sheath of the older leaf, chlorophyll accumulation does not commence until the new leaf has emerged (Friend, 1961). In cocoa, leaf developments proceeds by a pathway known as flush growth. The leaf expands to mature size and then synthesizes chlorophyll (Baker and Hardwick, 1973). After the cessation of leaf expansion, chlorophyll per unit area of leaf increases 2½ fold within 10 days. In the cucumber cotyledon, chlorophyll is rapidly formed on emergence from the soil, and reaches a maximum value after about 10 days (Becker et al, 1978). The synthesis of chlorophyll is therefore confined to specific periods of development according to the plant studied. Unlike chlorophyll, carotenoids are present in dark-grown leaves (Section I3A). In the light, the synthesis of carotenoids parallels chlorophyll synthesis in spinach (Lichtenthaler, 1968) and cocoa (Baker et al 1975).

The increased synthesis of thylakoid material during chloroplast development is accompanied by an increased synthesis of fatty acids for these membranes. Antirrhinum leaf fatty acid synthesis parallels chlorophyll synthesis during leaf development but falls behind during leaf expansion: linolenic (Radunz, 1966) acid and palmitic acid were found to be the predominant species. In maize, during early stages of leaf development, the synthesis of monogalactolipids, digalactolipids and sulpholipid is parallel with that of chlorophyll. Phosphatidyl glycerol, palmitic and linolenic acid synthesis lag

behind chlorophyll whilst the thylakoid component trans-hexadecanoic acid is found only in mature parts of the leaf. (Leech et al, 1973). In vitro, developing maize plastids incorporate [^{14}C]-acetate into fatty acids; the rate of this incorporation increased 5-fold with developmental age (Hawke et al, 1974). Palmitic and oleic acids were the major products; these plastids made no linolenic acid.

The development of proplastids into chloroplasts is accompanied by an increase in RNA content per leaf. Mung bean cotyledons show the major increase in cytoplasmic and chloroplast RNA content during the phase of cell division and leaf expansion (Grierson and Covey, 1975). The increase was considerably larger in the light than in the dark. Similar observations have been made using cucumber cotyledons (Becker et al, 1978). However, although there is an increase in rRNA content on a cellular basis in developing spinach leaves, the content per plastid decreases, although the chlorophyll content per plastid increases (Detchon and Possingham, 1972). Thus, rRNA synthesis is presumably slower than chlorophyll synthesis and does not keep pace with plastid division. In cotton cotyledons, where there is no cell division during the first 5 days of germination, there is an increase in chloroplast ribosome number and tRNA to 50% of the leaf total 5 days after germination (Brantner and Dure 1975). This is accompanied by an increase in chloroplast aminoacyl tRNA synthetases. Such an increase in the components of protein synthetic machinery is not unexpected in view of the increase in protein content of developing plastids.

During leaf expansion and development, there is an increase in chloroplast number per cell (Fasse-Franzisket 1956); Possingham and Saurer, 1970; Saurer and Possingham, 1969). This is accompanied by increased ct DNA synthesis. Rose et al (1974) have followed, by

autoradiography, the synthesis of chloroplast DNA in spinach leaf disks supplied with [^{3}H]-thymidine. All the chloroplasts were labelled during a 24 hour period. The dividing chloroplasts could be seen to pass the labelled ct DNA equally to daughter plastids during a cold chase procedure. In pea shoots grown in the light, plastid replication proceeds faster than DNA replication so that the DNA content per mature plastid is lower than in etioplasts. (Bennett and Radcliffe, 1975).

The controls of plastid division are not clear. In spinach, cell division in the secondary leaves continues until the leaves are between 1/3 and 1/2 full size. (Possingham and Saurer, 1970). Cell expansion can take place before and after cell division; chloroplast number keeps pace with cell division. In this system, plastid number was seen to increase by division of mature plastids. Thus, in this situation mature plastids may arise from two sources: from the proplastids and from other mature plastids.

The fraction of the cell space occupied by chloroplasts was found to be essentially constant and independent of cell size and age in spinach and tobacco (Honda et al, 1971). Butterfass (1973) has attempted to link the number of chloroplasts in a cell to its size and ploidy, and proposes that the plastid number is linked to the ploidy of the nuclear DNA. However, this is unlikely to be the sole control of plastid number because there are tissue-specific differences within a single plant.

The development of photosynthetic activity by plastids in plants grown under normal, diurnal light conditions does not occur at a fixed stage of leaf development. In pea, the maximum photosynthetic rate per leaf or per gram fresh weight of leaf is achieved 9 days after germination, which is during leaf expansion (Smillie, 1962). The maximum ribulose disphosphate carboxylase

activity per gram fresh weight is also achieved at this time. In Nicotiana, the maximum photosynthetic rate on a unit leaf area basis is also attained before the leaves reach the maximum size (Sestack, 1963). This observation contrasts with those of Dickman (1971), where the cottonwood tree was found not to attain the maximum photosynthetic rate until the leaves were fully expanded. This contrasts with the Cocoa flush growth, where in common with chlorophyll synthesis, the maximum rate is not achieved until after leaf expansion has ceased (Baker and Hardwick, 1973).

The general pattern of chloroplast development during growth under normal light conditions is therefore more gradual with respect to the onset of photosynthetic activity and the accumulation of chloroplast components than it is in etiochloroplast development. Etiochloroplast development represents the completion of certain light dependent steps of development in the partially mature plastid. Etioplasts contain many chloroplast components (Section I3A), the main exception being chlorophyll. In the more normal route of chloroplast development from proplastids, there is synthesis of many chloroplast components. It is also clear that the pattern of chloroplast development from proplastids is somewhat variable depending on the species examined. This variation appears less obvious in etiochloroplast development where the time scale of development is condensed.

The validity of etiochloroplast development has been questioned by a number of workers (Robertson and Laetsch, 1974; Kirk and Tilney-Bassett, 1978). The relationship between chloroplast, etioplast and proplastid can be described as triangular since proplastids can develop into either chloroplasts or etioplasts, depending on the availability of light. The dividing line between etioplast and proplastid is not marked during the early stages of development.

In bean, proplastids may enter a temporary state in which loose prolamellar bodies form (Whatley, 1974) while the primary leaves are still in the soil. Similar observations have been made by Leech et al (1972) using maize. In the youngest basal cells of the maize leaf, the majority of proplastids contained at least one prolamellar body. In these studies, however, the prolamellar body was a loose formation rather than the tight crystalline array observed in plants maintained in the dark for long periods of time. Kirk and Tilney-Bassett (1978) regard this latter etioplast as more common in the laboratory than in nature, since the conditions for the long periods of dark growth necessary for its formation would rarely occur in the wild.

I4. THE USE OF CELL-FREE SYSTEMS AS AN ASSAY FOR mRNA

Heterologous cell-free systems from a variety of sources have been used to translate mRNA fractions. The purpose of this section is to describe some of these systems and the features which are important in determining whether they are useful in the translation of particular mRNA molecules.

Cell-free extracts have been prepared from cells containing either 70S ribosomes, for example *E.coli*, or 80S ribosomes, for example wheat embryos, rabbit reticulocytes, Krebs II ascites and *Artemis* embryos. These systems will accept RNA from a wide range of sources, but the products obtained in each system may differ according to the properties of the system and of the RNA added.

Wheat germ extracts have been prepared by several methods (Roberts and Patterson, 1973; Marcus, 1971; Marcus *et al*, 1974; Zagorski, 1978); rye embryos have also been used to make cell free extracts (Carlier and Peumans, 1976). The advantages of the wheat germ system are that it is readily available and because of the dormant nature of the tissue contains a relatively low endogenous background of protein synthesis. Wheat germ extracts have been used to translate mRNA from a wide variety of sources: globin mRNA (Roberts and Patterson, 1973), rat liver preproalbumin mRNA (Taylor and Tse, 1976) brome mosaic virus (Shih and Kaesberg, 1973), cellulase mRNA (Verma *et al*, 1975) and *Euglena* mRNA (Sagher *et al*, 1975). Biologically active molecules have been synthesized in response to bacteriophage T3 RNA (Anderson *et al*, 1976) and T4 RNA (Beck and Gassen, 1977). However, such systems have a number of drawbacks. Added RNA can stimulate the endogenous protein synthesis in a wheat germ extract by as much as fourfold (Senger and Gross, 1976). In addition, some wheat germ extracts synthesize incomplete

chains in response to mRNA for polypeptides of high molecular weight (Tse and Taylor, 1977). This latter problem can be minimised by the addition of polyamines eg for tyrosine aminotransferase (Roewekamp et al, 1976) Tobacco Mosaic Virus (Hunter et al 1977) and collagen (Benveniste et al, 1976).

A further problem is the small amount of proteases and nucleases present in some wheat germ extracts (Davies and Kaesberg, 1973).

Translation systems based on 80S ribosomes have also been prepared from a variety of animal cells including reticulocytes (Lodish and Jacobson, 1972; Schrier and Staehelin, 1973; Pelham and Jackson, 1976) and ascites cells (Aviv et al, 1971; Mathews and Korner, 1970). The system of Lodish and Jacobson (1972) retained about 50% of the in vivo activity, and therefore there was a high level of endogenous globin synthesis. To use such active extracts to assay added mRNA they are treated with calcium-dependent micrococcal nuclease to remove endogenous mRNA, (Pelham and Jackson, 1976). However, care has to be exercised in this treatment since it can result in reduction of activity in the system, (Neelmam and Van Vloten-Doting, 1978). The advantages of the nuclease-treated reticulocyte lysate is that it synthesizes high molecular weight polypeptides with relatively few incomplete chains (Pelham and Jackson, 1976). In contrast, earlier studies with ascites extracts showed that incomplete polypeptide chains were synthesized in response to added mRNA (Boime and Leder, 1972).

To avoid the problems of in vitro assay, Xenopus laevis oocytes have been used to assay mRNA from a variety of sources, for example albumin mRNA (Zehavi-Willner and Lane, 1977), interferon mRNA (Colman and Morser, 1979) and albumin mRNA (Goodridge et al, 1979). The endogenous protein synthesis and pools of amino acids may present problems in detection of relatively minor mRNA species and only a

small amount of RNA can be injected into each cell. However, a major advantage of such a system is that it can carry out post-translational modifications to newly-synthesized polypeptides. Vitellogenin mRNA is translated and processed (Berridge and Lane, 1976); complete Tobacco Mosaic Virus coat protein is made (Hunter et al, 1976); immunoglobulin κ chain is processed to the mature size (Jilka et al, 1979); mouse interferon is synthesized as a biologically active molecule (Lebleu et al, 1978) and prostatic binding protein is glycosylated (Mous et al, 1979). Such activites are useful where there are post-translational modifications to polypeptides under study, but may not be essential for measurements of RNA amounts.

The only viable 70S ribosome translation systems available at the start of this project were those based on E.coli extracts (Modelell, 1971; Zubay, 1970). Such extracts have been used to translate chloroplast mRNA (Hartley et al, 1975; Gelvin et al, 1977; Howell and Gelvin, 1978) although Howell and Gelvin (1978) found that the large subunit of Fraction I protein was not synthesized as the full-sized polypeptide.

The spectrum of polypeptides synthesized in a cell-free extract in response to an mRNA preparation may depend on the structure of the mRNAs. Most eukaryotic mRNA species have a tract of polyadenylic acid (poly(A)) at the 3' end; this tract can be anything between 50 and 200 residues in length. The major exceptions are histone and reovirus mRNAs which contain no detectable poly(A) or only some poly(A) of limited length (Greenberg and Perry 1972; Stoltfus et al, 1973). Plant cytoplasmic polysomal RNA also contains poly(A) (Van derWalle, 1973; Sagher et al, 1974). Chloroplast mRNA does not bind to oligo(dT) under conditions which would allow only stretches of more than about 20 adenylate residues to bind (Wheeler and Hartley,

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1975). It has been reported that the mRNA for the large subunit of Chlamydomonas Fraction I protein contains no poly(A) (Sagher et al, 1975) or small stretches of variable length (Sano et al, 1978). This situation contrasts with the finding that both mitochondrial mRNA (Haff and Bogorad, 1975) and bacterial mRNAs (Srinivassan et al 1975; Ohta et al, 1975) contain short poly(A) tails.

Since mRNA lacking poly(A) can be translated in cell-free extracts (Sagher et al, 1975; Sano et al, 1979) it is of interest to find out the function of these poly(A) structures. One suggested function of poly(A) is to stabilize mRNA against degradation (Darnell et al, 1973). Perry and Kelly (1974) examined the degradation of mRNA with age and found that there was no preferential degradation of any particular age class of mRNA. Since the poly(A) tail shortens during the lifespan of mRNA, it was assumed that shorter poly(A) tails do not necessarily result in shorter mRNA lives. Sheiness et al (1975) found that there was a minimum length of poly(A) attached to mRNA. They argued that this could result from the random cleavage of poly(A) tails, if tails of less than 50 residues length are degraded.

However, the evidence in favour of the stability function has come from in vivo studies. Enzymic removal of poly(A) from mRNA does not affect its translation in a cell-free system (Williamson et al, 1974; Bard et al, 1974; Doel and Carey, 1976; Humphries et al, 1974). However, such systems only initiate protein synthesis for a limited period of the assay; if Xenopus oocytes are used, the long term effect of poly(A) removal can be examined. In such a system globin mRNA is translated with or without poly(A) tails but the mRNA lacking poly(A) is degraded more rapidly than that having poly(A) (Marbaix et al, 1975; Huez et al, 1974).

Nudel et al (1976) have shown that reduction of poly(A) tails to 32 residues in length reduces the stability of mRNA; below 16 residues, the stability of the mRNA was the same as that without any poly(A). It is possible to restore stability by readenylating mRNA (Huez et al, 1975). Adenylation of 5S RNA, which does not normally contain poly(A), also confers stability if the poly(A) tail is greater than about 20 residues in length (Heiter et al, 1976). Poly(G) is also effective in this role. The length of the poly(A) tail on EMC virus mRNA affects its stability in an Erlich ascites cell-free extract (Hruby et al 1978) when assay is carried out for longer time periods.

Thus, it appears that poly(A) tails may confer a long-term stability on a mRNA species. With respect to the in vitro translation of mRNAs lacking such structures in the cell-free systems from wheat germ or reticulocytes, the lack of polyadenylation is not a block to translation.

A major feature of the 5' terminus of many eukaryotic and viral mRNAs is a structure known as the cap. The structure and function of caps have been comprehensively reviewed by Shatkin (1976) and Filipowicz (1978). The cap may be represented generally as $m^7G^{(5')}ppp^{(5')}X^{(m)}pY^{(m)}$ where X and Y are any of the four bases, X usually being A or G. Some viruses, including Vaccinia (Wei and Moss, 1974) reo (Furuichi et al, 1976) and vesicular stomatitis virus (Rhoads et al, 1974) encode enzymes which cap viral RNA.

The cap structure is now thought to have two major functions: to facilitate the formation of the initiation complex between mRNA and the ribosome, and to protect the mRNA against degradation (Filipowicz, 1978). The former function is of especial relevance in the translation of mRNA in a heterologous cell-free extract.

The function of and requirement for cap structures have been elucidated by translation studies on mRNAs in cell-free extracts. Two approaches have been used to show that a cap is required for efficient translation of mRNA in wheat germ extracts. When capped and uncapped VSV, reovirus and globin mRNAs are presented to the wheatgerm system, only the capped mRNAs are translated efficiently (Muthukrishnan et al, 1975; Kemper, 1976). Similarly, if cap analogues are added to the system, the binding of capped mRNAs to ribosomes is impaired (Canaani et al 1976, Shafritz et al, 1976; Hickey et al, 1976). It is clear that this effect is partially due to prevention of the formation of initiation complexes (Roman et al, 1976). Such effects of caps on initiation are more obvious in wheat germ extracts than in reticulocyte lysate extracts (Lodish and Rose, 1977). The apparent importance of caps in translation also depends on the conditions of assay. Efficiency of translation of uncapped mRNAs can be improved by increased mRNA concentration (Shih et al, 1976; Hickey et al, 1976) or by changing ionic conditions. The dependence upon caps of translation is less marked at low K^+ concentration (40-80 mM) (Weber et al, 1977a) than at high K^+ concentrations (Weber et al, 1977b; Wodnar-Filipowicz et al, 1978; Weber et al, 1978; Kemper and Stolarskry, 1977). Recently, Bergman and Lodish (1979) tested VSV (Vesicular Stomatitis Virus.) and reovirus RNA with and without caps in reticulocyte lysate and wheat germ extracts, and showed that the uncapped mRNAs have a lower K^+ optimum than the capped species. It was also clear that the cap requirement was less stringent in reticulocyte lysate extracts compared to wheat germ extracts.

The role of caps in mRNA stability has been demonstrated in Xenopus, L cell and wheat germ extracts (Furuichi et al 1977). In these systems, VSV RNA with a variety of cap structures are less

rapidly degraded than those containing no cap structures. A similar effect has been found on the stability of Cowpea Mosaic Virus mRNA in wheat germ extracts (Shimotohno *et al*, 1977). However, the rabbit reticulocyte lysate does not contain nucleases which degrade uncapped reovirus mRNAs (Furuichi *et al*, 1977). The situation is made more complex by the finding that some decapped mRNAs are more stable than others. Decapped TMV RNA is more stable than decapped globin mRNA in wheat germ extracts (Wodnar-Filipowicz *et al*, 1978). This effect may be due, in part, to the presence of proteins in some cell-free extracts eg *Artemia* (Filipowicz *et al*, 1976) which bind caps.

It is possible to artificially cap prokaryotic, uncapped mRNAs eg. λ *cro* mRNA so that their translation is as efficient as capped, eukaryotic mRNAs (Paterson and Rosenberg, 1979) in wheat germ extracts. However, the lack of a cap may not be the only block to the translation of prokaryotic mRNAs in eukaryotic systems. Herrlich and Schweiger (1978) go as far as to state "There is no efficient translation of heterologous mRNA". A comparison of translation of eukaryotic and prokaryotic RNA preparations *in vitro* in extracts of wheat germ, Krebs ascites cells, reticulocytes and *E.coli* showed that only homologous translation was efficient (Herrich and Schweiger, 1978). This was found to be due to lack of initiation, perhaps due to a lack of appropriate homologous sequences in the initiation region. Both (1979) has extended the importance of such base pairing to interactions within the coding regions of mRNAs.

In prokaryotes, a pyrimidine-rich region conserved in the 16S rRNA base-pairs with purine-rich sequences in mRNAs which are

located 5'-proximal to the AUG, (Shine and Dalgarno, 1975; Steitz and Jakes, 1975); such sequences may stabilize initiation complexes. Similar interactions have been proposed for eukaryotic mRNA and ribosomes (Hagenbuchle *et al*, 1978). However, such complementarity is not universal; the complementarity must also be flexible because it is possible to obtain translation of mRNAs with good efficiency in heterologous systems.

The choice of cell-free extract in the assay of mRNA is therefore to some extent, governed by the mRNA under study. For the translation of chloroplast mRNA *in vitro*, a 70S system such as that based on *E.coli* ribosomes might seem, at first sight, to be most useful. However, such a system has been found to give variable results (Howell and Gelvin, 1978; Hartley *et al*, 1975; Sano *et al*, 1979) and is therefore not suitable for quantitative assay of mRNA. Thus it is necessary to turn to eukaryotic systems. Since chloroplast RNA is prokaryotic in nature, it is unlikely to be capped. It is therefore necessary to use an extract such as the reticulocyte lysate which does not greatly discriminate against uncapped mRNAs. Such a system has the added advantages of low endogenous protein synthetic activity (Pelham and Jackson, 1976) and reproducibility.

I5 AIMS OF THE PRESENT WORK

This introductory review has indicated that chloroplasts contain DNA and a protein-synthetic machinery which synthesize a limited spectrum of chloroplast polypeptides. (Section I2 B and C.) Intact isolated chloroplasts incorporate radioactive amino acid into at least 90 polypeptides (Ellis et al, 1978) and it is of interest to know whether these polypeptides are synthesized at all stages of chloroplast development or in a non-coordinate fashion. However, since most of the structural genes located in the chloroplast genome are largely unidentified, it is not possible to ask the direct question "which genes are switched on, and in what order?". This question was therefore tackled using the more indirect approaches available at the start of this project.

The strategy for studying the control of chloroplast gene expression followed in this project may be divided into three parts:-

1. to gather information about the growth and development of the tissue under study, for example leaf size, chlorophyll and Fraction I protein content;
2. to label chloroplast proteins both in vivo and in isolated chloroplasts, and to measure the relative incorporation of radioactivity into the major chloroplast polypeptide products;
3. to assay the amount of translatable mRNA for these chloroplast polypeptides in a heterologous cell-free extract.

A number of developmental systems were available for the study of chloroplast development at the start of this project. As reviewed in Section I3, chloroplast development has mainly been studied from etioplasts. This developmental pathway is not a common one in nature

so it was decided to examine chloroplast development under a diurnal light regime.

Spinach (Spinacia oleracea var. Monstrous Viroflay) was chosen as the plant tissue for this study for a number of reasons. It has been used for studying protein synthesis in isolated chloroplasts (Bottomley et al, 1974; Mendiola-Morgenthaler et al, 1976) and spinach RNA can be translated in a heterologous cell-free system (Hartley et al, 1975; Bottomley et al, 1976). A major advantage of the spinach plant is that the primary leaf pair grows in a regular and reproducible fashion. Such a leaf is easily grown and used for developmental studies. This tissue contrasts with the pea apex which represents a collection of leaves at heterogeneous stages of development.

Initially, it was decided to grow the spinach plants in compost. However, such plants did not yield chloroplasts active in protein synthesis in vitro. Plants were therefore grown in hydroponic culture for this purpose and the data compared on the basis of growth parameters such as leaf length.

At the start of this project, few chloroplast structural genes had been identified, so it was decided to concentrate on the synthesis of two known products of chloroplast protein synthesis, LSU and peak D. Prior to the present study, it had been shown that during the light-induced greening of pea etioplasts, the relative amount of synthesis of LSU and peak D changes as greening proceeds (Siddell and Ellis, 1975). Isolated pea etioplasts synthesize LSU but not peak D; as greening proceeds, plastids isolated from the leaves

synthesize peak D to an increasing extent, until it becomes the dominant product. The present study offered the opportunity to test whether this change in protein synthesis occurs in plants grown under a diurnal light regime and whether this change in gene expression reflects changes in the translatable mRNA for these two polypeptides.

The heterologous cell-free translation systems currently available have been reviewed in Section I4. Although spinach chloroplast RNA had been successfully translated in an E. coli cell-free system (Hartley et al, 1975), the reticulocyte lysate was chosen for quantitative studies because of its low endogenous synthesis.

In order to avoid the problems of quantitation of the absolute amounts of LSU and peak D synthesized in vivo, in isolated chloroplasts and in the reticulocyte lysate, changes in incorporation were expressed as a ratio throughout development. A comparison of the magnitude of the ratio LSU: peak D in the three systems chosen for study should indicate at what level the expression of chloroplast DNA is controlled.

SECTION II - MATERIALS AND METHODS

1. MATERIALS

A. Plant material

Spinach seeds (Spinacea oleracea var. Monstrous Viroflay) were obtained from Thompson and Morgan Ltd., Ipswich, Suffolk, U.K.

(i) Compost-grown plants

Seeds were immersed in 2% (v/v) hypochlorous acid and swirled continuously for 5 minutes. A volume of 500 ml hypochlorous acid was used for each 100 ml packed volume of seeds. The acid was then poured off, and the seeds rinsed thoroughly with tap water until no traces of acid remained. Washed seeds were spread out thinly on a sheet of Whatman 3 MM paper in a tray, presoaked with tap water. The edges of the paper were dipped in a shallow reservoir of water to prevent drying out during germination, and the whole tray placed in a dark, ventilated cupboard. After 3 days, seeds showing signs of germination (here defined as radicle emergence) were transplanted into compost. Ward seed trays (35 x 21.5 cm) were filled to a depth of 3 cm with moist compost (J. Arthur Bowers compost from Lindsay and Kestevens, Ltd., Saxilby, Lincoln) and the seeds planted out at 2.4 cm intervals, allowing a margin around the edge of the tray. Each tray contained about 70 seeds. After overlaying with a further 2 cm of moist compost, the trays were placed under illumination of 10 000 lux from warm-white fluorescent tubes. The photoperiod was 9 hours light and 16 hours darkness and growth was continued in a well-ventilated room at a temperature of 20-22^{o} C. The plants were watered daily with tap-water.

(ii) Hydroponically-grown plants

Sterilized seeds were sown in moist compost as described above at a density of 50 ml seeds per tray. After 7 days the seedlings were carefully removed from the soil, their roots washed

in tap-water and the plants then transferred to liquid culture. Trays (13.8 x 26.5 cm, depth 8.5 cm) were filled to within 2 cm of the top with Huntner's medium (Huntner, 1953) and a grid drilled with holes 0.9 cm in diameter, spaced at 1.6 cm intervals, in a 9 x 5 arrangement, placed on top. Each seedling was wrapped around the stem with foam rubber and placed in the grid. Trays were kept under the same regime as the compost trays, except that continuous aeration was also supplied to the solution.

B. Chemicals, biochemicals and radiochemicals

All materials used were of Analar or equivalent grade. The source of specific chemicals and biochemicals is given below.

Sigma (London) Chemical Co. Ltd., Dorset, U.K.

Chemicals:- L-amino acids, chloroamphenicol (D-threo isomer), Coomassie Brilliant Blue R250, creatine kinase, creatine phosphate, cycloheximide, cysteine, ethylene glycol tetraacetic acid (EGTA), haemin, heparin (sodium salt), sodium isoascorbate, micrococcal nuclease, 2-(N-morpholino) ethane sulphonic acid (Mes), phenylhydrazine, phenylmethylsulphonyl fluoride (PMSF), sorbitol, N-tris (hydroxymethyl) methyl glycine (Tricine), 2-amino-2-hydroxymethyl propane-1,3-diol (Tris, "TRIZMA" base), octyl phenoxy polyethoxyethanol (Triton X-100).

Proteins:- bovine serum albumin (BSA), carbonic anhydrase, chymotrypsin, cytochrome c, hemoglobin, myoglobin, ovalbumin, soybean trypsin inhibitor, trypsin.

B.D.H. Chemicals Ltd., Dorset, U.K.

Acrylamide, ammonium persulphate, boric acid, bromophenol blue, chloroform, m-cresol, dimethylsulphoxide (DMSO), ethylene glycol (ethanediol), Folin-Ciocalteau reagent, glycine, linear polyacrylamide, DL-methionine, 2-mercaptoethanol, sodium hydroxide

(CVS solution), sodium dodecyl sulphate (SDS), trichloroacetic acid.

Fisons Scientific Apparatus, Leics., U.K.

8-hydroxyquinoline, hydrogen peroxide (100 vol.), isoamyl alcohol, phenol (80% (v/v) liquified AR), 2,5-diphenyl oxazole (PPO).

Koch-Light Ltd., Bucks, U.K.

Ethylene diamine tetraacetic acid (EDTA).

Hopkin and Williams Ltd., Essex, U.K.

N-2-(hydroxyethylpiperazine-N-yl) ethane sulphonic acid (Hepes).

Eastman Kodak, Rochester, New York, U.S.A.

N,N'-methylene bisacrylamide (bisacrylamide), Kodirex X-ray film, N,N,N',N'-tetramethylethylene diamine (TEMED).

Pharmacia (Great Britain) Ltd., London, U.K.

Sephadex G25 and Sephadex G200.

Whatman Ltd., Kent, U.K.

Whatman papers, DEAE cellulose (DE52).

Radiochemical Centre, Amersham Bucks, U.K.

L-[^{35}S]methionine (800-1000 Ci/mmol).

Nuclear Enterprises, Edinburgh, U.K.

1,4-bis-(5-phenyl oxazole-2-yl)benzene (POPOP).

Cambrian Chemicals, Surrey, U.K.

Worthington DNAase I (RNAase-free).

Du Pont (U.K.) Ltd., Hunts, U.K.

Cronex film.

Miles Laboratories Ltd., Bucks, U.K.

Staphylococcus aureus V8 protease

2. METHODS

A. Preparation of soluble and insoluble protein fractions from whole spinach primary leaves

The following procedure was used to prepare a soluble protein fraction for Fraction I protein estimation (Section II 2B(ii)) and to fractionate soluble and insoluble *in vivo*-labelled proteins (Section II 2C). All operations were carried out at 0-4° C with sterile equipment and solutions where possible, to minimise proteolysis by microorganisms.

Primary leaves, excised at the leaf base, were placed in a chilled mortar and ground in a minimal volume (1.0 ml/0.5 g leaves) of a buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA. Additions of 10 mM 2-mercaptoethanol and 2 mM PMSF were made to this buffer just before use from concentrated stock solutions of 11 M and 200 mM in DMSO respectively. PMSF is an effective inhibitor of serine proteases and has been demonstrated to prevent proteolysis of Fraction I protein in *Phaseolus* extracts (Gray and Kekwick, 1974). Where the tissue was labelled with L-[^{35}S]-methionine before grinding, saturated DL-methionine was added to the grinding medium (100 µl saturated DL-methionine /ml). The tissue was ground in the presence of glass beads (80 mesh) until no particulate material was visible, and the homogenate decanted. The pestle and mortar were washed out with further grinding buffer, and the wash added back to the original homogenate. A supernatant solution containing soluble proteins was obtained by centrifugation of the homogenate at 30 000 g for 10 minutes. The supernatant solution was retained on ice, whilst the pellet was resuspended in a minimal volume of grinding buffer and again centrifuged at 30 000 g. The original and wash supernatant fractions were combined and, if applicable, were used for estimation of Fraction I protein content.

Where leaves had been labelled *in vivo* prior to grinding, the final pellet was resuspended in a volume of grinding buffer equivalent to the supernatant volume. Aliquots were taken from soluble and insoluble protein fractions for estimation of trichloroacetic acid-insoluble incorporation (Section II 2C). The remaining samples were adjusted to a final concentration of 2% (w/v) SDS, boiled for 2 minutes, and the proteins analysed on SDS-polyacrylamide gels (Section II 2F(b)).

B. Estimation of leaf Fraction I protein content

(i) Preparation of pure Fraction I protein

Pure Fraction I protein was obtained by the method of Blair and Ellis (1973) which is a modification of the procedure of Kawashima and Wildman (1971). All operations were carried out in the cold, and equipment and solution sterilised where possible to minimise proteolytic degradation.

Mature spinach leaf tissue (200 g) was harvested from 70 day-old plants, and the leaf midribs removed with sharp scissors. The tissue was homogenized in 300 ml ice-cold Buffer A (25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 40 mM 2-mercapto-ethanol, 2 mM PMSF) in an Atomix blender for 1 minute at top speed. After filtration through 8 layers of muslin to remove debris, the homogenate was centrifuged at 10 000 g for 10 minutes, followed by 30 000 g for 60 minutes, to yield a yellow, high speed supernatant solution. Low molecular weight material was removed from this supernatant liquid by passing it down a column (6.6 x 50 cm) of coarse Sephadex G25 washed with Buffer B (25 mM Tris-HCL (pH 7.4), 50 mM NaCl, 0.5 mM EDTA, 10 mM 2-mercaptoethanol). The brown fraction which eluted with the void volume was collected. A 35-45% saturated ammonium sulphate cut was taken by gradual addition of the solid, with stirring for 30 minutes, and precipitates

collected by centrifugation at 10 000 g for 10 minutes. The material precipitated between 35% and 45% saturation was resuspended in a minimal volume of Buffer C (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM 2-mercaptoethanol) and dialysed overnight against 1 litre of Buffer C.

The yellow dialysed solution was placed on a column (1.5 x 15 cm) of DE 52 cellulose previously equilibrated with Buffer C. When no further material absorbing at 280 nm could be washed off with Buffer C, the column was washed with Buffer C containing 100 mM NaCl. Material absorbing at 280 nm was collected, and the protein precipitated by the addition of ammonium sulphate to 50% saturation and pelleted by centrifugation at 10 000 g for 10 minutes. The pellet was resuspended in 5 ml Buffer B and loaded on to a column (2.5 x 90 cm) of Sephadex G200, previously equilibrated with this buffer. Protein was eluted with Buffer B at a flow rate of 2.5 ml/h, and 2 ml fractions collected. Fractions having A280: A260 ratios of 1.8 or greater were pooled, the protein precipitated by addition of ammonium sulphate to 50% saturation, and collected as described above.

The purified Fraction I protein pellet was resuspended in Tris-glycine buffer (2.5 mM Tris-HCl (pH 8.5), 19.2 mM glycine, 10 mM 2-mercaptoethanol) and dialysed against 1 litre of this buffer overnight. The protein was stored as a freeze-dried powder at -20° C.

(ii) Estimation of Fraction I protein levels by dye binding.

Native Fraction I protein prepared by the method in Section II 2B (i) was used as the standard protein to calibrate the dye-binding procedure. The freeze-dried powder was dissolved in Fraction I protein buffer (2.5 mM Tris-HCl (pH 8.5), 19.2 mM glycine, 10 mM 2-mercaptoethanol, 0.001% bromophenol blue, 5% (w/v) sucrose) to a

final concentration of 1 mg / ml just before use. The soluble protein supernatant fractions to be used for estimation (Section II 2A) were adjusted to 0.001% bromophenol blue, 5% (w/v) sucrose (final concentrations) and retained on ice.

Non-denaturing cylindrical gels containing 6% (w/v) polyacrylamide (Section II 2F (ii)a) were loaded with a range of 0-150 µg Fraction I protein standard and a similar range of protein concentrations in the supernatant fraction to be tested. Electrophoresis was performed for 3 hours at 5 mA constant current per gel at room temperature. After this period, the bromophenol blue dye just reaches the bottom of the gel. After electrophoresis, the gels were fixed in 7% (v/v) acetic acid for 1 hour and then stained in 0.5% (w/v) amido black dye in 7% (v/v) acetic acid. Destaining was carried out electrically at 1 amp per 8 gels for 1 hour in 7% (v/v) acetic acid. The Fraction I protein standard gels contained a single, heavily-stained band 1-2 cm from the top of the gel. These bands, and the bands of corresponding mobility from the supernatant samples were chopped out, using a razor blade to yield slices of gel 0.5 cm or less in thickness. Corresponding slices from blank gels were introduced to provide blank readings. Each slice was transferred to 2 ml sodium hydroxide (1 M) and incubated at 37^{o} C for 16 hours to elute the dye. Incubation was carried out in the dark since light causes the dye to fade. The absorbance of each sample at 615 nm was read against the gel blank and a calibration curve constructed from the Fraction I protein standard points. This curve was used to estimate the amount of Fraction I protein in the tissue sample.

C. In vivo labelling of spinach primary leaves with [^{35}S]-methionine

Excised spinach leaves were labelled by feeding radioisotope in solution through the cut end, by means of the following procedure. Developing spinach primary leaves were excised at the base of the petiole with a razor blade, and the cut end immediately dipped into 0.5 ml sterile distilled water containing 100 µCi L-[^{35}S]methionine (850-1000 Ci/mMol). Variable numbers (5-10) of leaves were incubated in each pot, depending on the age of the leaves. For young leaves of less than 1.5 cm length from tip to base, incubation was carried out in the wells of a haemagglutination tray with support given by a plastic collar around the well opening. Older leaves were incubated in Unicam vials. The leaves were incubated for 6 hours at 20-22° C under 10 000 lux illumination from warm-white fluorescent tubes. If the feeding solution was all taken up within this period, further sterile distilled water was added to the incubation container.When inhibitors of protein synthesis were used, a concentrated stock was made up in sterile distilled water and added to the feeding solution to final concentrations of 50 µg/ml (D-threo chloramphenicol) or 2 µg/ml (cycloheximide). After the labelling period was completed, the leaves were blotted dry on tissue, the petioles discarded, and the leaves homogenized as described in Section II 2A.

Hot trichloroacetic acid-insoluble incorporation was estimated from small duplicate aliquots, usually 10 µl, of the soluble and insoluble protein fractions. Each aliquot was delivered into 100 µl of a BSA solution (1 mg/ml) and precipitated by the addition of 3 ml ice-cold 5% (w/v) trichloroacetic acid. The samples were incubated at 4° C overnight to allow precipitation and flocculation of the protein. The precipitates were then heated to 90° C for 10

minutes to destroy methionyl-tRNA complexes charged with radioactive methionine which could lead to overestimation of incorporation. The cooled samples were filtered under vacuum and the precipitates collected on 2.5 cm Whatman GF/C filters which had been presoaked in 5% (w/v) trichloroacetic acid. Each filter was washed with a total of 40 ml 5% (w/v) trichloroacetic acid followed by 10 ml ethanol. The filters were dried at 50° C for 30 minutes, cooled, and their radioactivity measured in a Packard Tricarb liquid scintillation spectrometer in 4 ml toluene-based scintillant (0.5% (w/v) PPO, 0.03 (w/v) POPOP).

D. Preparation and incubation of isolated spinach chloroplasts

(i) Preparation of chloroplasts by differential centrifugation

Plastids active in protein synthesis were isolated by the differential centrifugation method of Blair and Ellis (1973). Sterile solutions and equipment were used where possible to minimise contamination by microorganisms.

Spinach primary leaves (15 g) were homogenized for two bursts of 4 seconds in a Polytron homogenizer (Northern Media Supplies, Hull, U.K.) set at speed 7 in 100 ml semi-frozen sucrose isolation medium (0.35 M sucrose, 25 mM Hepes-NaOH (pH 7.6), 2 mM EDTA, 2 mM sodium isoascorbate). The resultant homogenate was strained through 8 layers of muslin and centrifuged at 2500 g for 1 minute at 0° C. The supernatant solution was poured off and the tube walls wiped with tissue to remove most of the remaining liquid. Pellets were resuspended in sucrose isolation medium (not semi-frozen) with a cotton-wool bud, and suspensions again centrifuged at 2500 g for 1 minute at 0° C. The supernatant solution was poured off, the walls of the tubes wiped with tissue and the pellets resuspended in a buffer appropriate to their subsequent treatment (Sections II 2D (ii) and (iii)).

(ii) Incubation of isolated chloroplasts

The procedure used was based on that of Blair and Ellis (1973) using the sorbitol-based incubation medium of Bottomley et al. (1974).

Plastids were isolated as described in Section II 2D (i) and the pellets resuspended in 0.33 M sorbitol, 50 mM Tricine-KOH (pH 8.4). Examination of this suspension in a phase contrast microscope revealed that the preparations were normally at least 60% intact as judged by phase-brightness (Siddell and Ellis, 1975). Aliquots (1 ml) of plastid suspension were incubated with 100 µCi L-[^{35}S]methionine at a final chlorophyll concentration of 100-400 µg/ml. Incubation was carried out in a Churchill water bath, maintained at 20° C with illuumination underneath supplied by a photoflood bulb (PF2, Phillips, U.K.). The light intensity at the edge of the tube was at least 50 000 lux as measured by a Megatron light meter, type E1.

After 45 minutes, incubations were terminated by the addition of 100 µl of a saturated DL-methionine solution and the tubes transferred to ice. At this stage, duplicate aliquots (10 µl) were removed for estimation of trichloroacetic acid-insoluble incorporation as described in Section II 2C. The remaining samples were adjusted to a final concentration of 2% (w/v) SDS from a 20% (w/v) SDS stock solution, boiled for 2 minutes, and then analysed by SDS-polyacrylamide gel electrophoresis (Section II 2F (i)b).

(iii) Preparation of total RNA from isolated chloroplasts

The method used for preparation of chloroplast RNA was based on that of Brawerman (1974). Phenol and SDS were used to extract total nucleic acid, the DNA portion being removed by a subsequent treatment with deoxyribonuclease (DNAase). Plastid pellets prepared as described above (Section II 2D (ii)) were resuspended

in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2% (w/v) SDS with a sterile Pasteur pipette. An equal volume of phenol reagent was added, the tube covered and shaken vigorously. The phenol reagent contained phenol/8-hydroxy-quinoline/m-cresol (500 g/0.5 g/70 ml respectively) mixed 50:50:2 (by volume) with chloroform and isoamyl alcohol respectively. After 10 minutes standing at room temperature, the mixture was separated into two solvent phases by low-speed centrifugation. The upper aqueous layer was decanted and retained whilst the phenol phase was re-extracted with a half volume of the SDS extraction buffer. The aqueous phases were pooled and re-extracted two times more with an equal volume of phenol. At this stage, no protein was visible at the interface between the phenol and aqueous phases. The nucleic acids in the final aqueous phase were precipitated by the addition of two volumes of redistilled ethanol, followed by overnight storage at -20° C.

The DNA was removed as follows. The precipitated nucleic acids were collected by centrifugation at 3000 g for 10 minutes, and the pellet washed in 70% (v/v) aqueous ethanol containing 50 mM NaCl, to remove any SDS remaining from the phenol extraction. The pellet was dried under nitrogen and then resuspended in 50 mM Mes-NaOH (pH 7.0), 2.5 mM magnesium acetate. DNAase I (Worthington, ribonuclease-free) from a 1 mg/ml stock in DNAasing buffer was added to a final concentration of 20 µg/ml, and the tubes incubated for 15 minutes on ice. After adjustment to 100 mM with respect to NaCl, the RNA was reprecipitated by the addition of 2 volumes of redistilled ethanol, followed by overnight storage at -20° C.

Before use, the RNA was reprecipitated once from lithium acetate buffer (0.15 M lithium acetate-acetic acid (pH 6.0) 0.5% (w/v) SDS), and at least twice from 250 mM Tris-HCl, pH 7.8. The

RNA was then collected by centrifugation, the pellet dried under nitrogen and dissolved as appropriate to its subsequent use.

E. The cell-free protein-synthesizing system from rabbit reticulocytes

(i) Preparation

The lysate was prepared by Dr. John Morser in the Virus Laboratory of this Department. Six male New Zealand White rabbits, each weighing between 2 and 2.5 kG, were injected daily with 0.6 ml phenylhydrazine (0.25% w/v) for 5 days to make them anaemic. After one day of rest, the rabbits were bled by cardiac puncture into a heparinised syringe and the blood transferred into a heparinised glass beaker. The reticulocytes were collected by centrifugation and washed three times in a solution containing 0.145 M NaCl, 50 mM KCl, 5 mM $MgCl_2$. The pellet obtained was then lysed in 1.5 volumes of cold, sterile distilled water and the resultant lysate centrifuged at 10 000 g at 0° C for 5 minutes to remove cellular debris. The supernatant liquid was stored frozen in 1 ml aliquots in liquid nitrogen.

Nuclease treatment of the lysate was carried out by the method of Pelham and Jackson (1976). A 1 ml aliquot of lysate was removed from liquid nitrogen and immediately made 50 µg/ml in creatine kinase and 25 µM in haemin from stock solutions of 5 mg/ml creatine kinase in 50% (v/v) aqueous glycerol and 1 mM haemin in 90% (v/v) aqueous ethylene glycol, 20 mM Tris-HCl (pH 8.2), 50 mM KCl. After thawing was complete, the lysate was made 1 mM with respect to calcium chloride and 10 µg/ml with respect to micrococcal nuclease, from stock solutions of 100 mM calcium chloride and 1 mg/ml nuclease in sterile distilled water respectively. The tube was rolled to give complete mixing and then incubated 15 minutes at 20° C. Micrococcal nuclease is calcium-dependent, so to terminate the nuclease treatment

calcium was chelated with a final concentration of 2 mM EGTA (ethylene glycol tetraacetic acid) from a 50 mM EGTA stock (neutralised with KOH). After mixing, the lysate was stored in 100 µl and 200 µl aliquots under liquid nitrogen.

(ii) Use as an assay for translatable mRNA

(a) Preparation of incubation mixtures and estimation of trichloroacetic acid-insoluble incorporation

Total chloroplast RNA preparations were assayed for translatable mRNA by incubation with nuclease-treated reticulocyte lysate in the presence of L-[^{35}S]methionine. Incubation of final volume 20 µl were carried out in conical plastic vials (500 µl nominal capacity, Sarstedt (U.K.) and washed as described in Section II 20 (iii) and finally dissolved in sterile distilled water to a suitable final concentration. RNA concentrations were calculated on the basis that 1 mg/ml RNA is equivalent to 20 A_{260} units.

The reticulocyte lysate incubation mixtures contained the following components at the final concentrations listed:-

reticulocyte lysate (nuclease-treated), 75% (v/v); creatine phosphate, 10 mM;

all amino acids except methionine at the concentrations found in globin, (Hunt and Jackson, 1974);

potassium chloride, 75 mM;

magnesium acetate, 0.5 mM;

L-[^{35}S]methionine (850-1000 Ci/mMol), 5 µCi.

These concentrations refer to the added materials and do not include the salts and amino acids already present in the reticulocyte lysate. Solutions were stored in small aliquots at -90^{o} C. The incubations were made up to volume by the addition of sterile distilled water where necessary. During making up of incubations, all solutions

were kept on ice, and the lysate was thawed and added last. Incubation was carried out at 30° C for 60 minutes, except during time-course experiments.

Trichloroacetic acid-insoluble incorporation of [^{35}S]-methionine was assayed from duplicate 2 µl aliquots of each incubation mixture. These aliquots were spotted on to 2 x 1 cm strips of Whatman No. 1 filter paper (Bollum, 1968). The strips were dried and heated to 90° C for 20 minutes in 10% (w/v) trichloroacetic acid containing 0.5% (w/v) DL-methionine and 5% (v/v) hydrogen peroxide (100 vol.) to bleach the samples (at least 10 ml/strip). The strips were then washed with 10% (w/v) trichloroacetic acid containing 0.5% (w/v) DL-methionine (at least 5 ml/strip) and finally washed with diethyl ether (at least 5 ml/strip). After drying with a fine stream of nitrogen, the strips were dried completely at 70° C and measured for radioactivity in toluene scintillant (0.5% (w/v) PPO, 0.03% (w/v) POPOP) in a Packard Tricarb liquid scintillation spectrometer.

(b) Analysis of products on SDS polyacrylamide gels

The remaining volumes of incubated samples were analysed on SDS polyacrylamide gels after preparation by the method of Highfield (1978). The remaining sample was adjusted to a final volume of 100 µl containing final concentrations of 2% (w/v) SDS, 100 mM 2-mercaptoethanol, 5% (w/v) sucrose, 0.05% (w/v) bromophenol blue and 1 x Neville Upper Reservoir Buffer (Neville, 1971). Samples were boiled for 2 minutes to fully denature the protein and then analysed on polyacrylamide gels by the method described in Section II 2F (i)b.

F. Protein and RNA analysis by polyacrylamide gel electrophoresis

(i) Protein gels

(a) Non-denaturing cylindrical gels

Non-denaturing gels containing 6% (w/v) polyacrylamide were run by the method of Blair and Ellis (1973). Cylindrical gels 9 cm in length were cast in Perspex tubes (0.6 x 10 cm). Each gel contained a final concentration of 0.375 M Tris-HCl (pH 8.5), 6% (w/v) acrylamide and 0.12% (w/v) bisacrylamide from stock solutions of 3.0 M Tris-HCl (pH 8.5) and 40% (w/v) acrylamide, 0.8% (w/v) bisacrylamide respectively. The gel mixture (40 ml) was degassed with a vacuum line and TEMED and ammonium persulphate (0.06% (w/v) and 0.07% (w/v) respective final concentrations) added to effect polymerisation. Gel tubes, closed off at one end with moist dialysis film, were filled to the desired level with gel solution and then overlaid with water to give a smooth loading surface. Polymerisation took 15-20 minutes. The polymerised gels were then electrophoresed for 1 hour at 10 mA per gel (constant current) to remove remaining persulphate. Samples prepared as described in Section II 2A were made 5% (w/v) in sucrose and 0.05% (w/v) in bromophenol blue and then loaded on to the gels. Electrophoresis was performed for 3 hours at 5 mA per gel in electrophoresis buffer (50 mM Tris-HCl (pH 8.5), 380 mM glycine. The upper (cathode) reservoir buffer additionally contained 8 mM cysteine-HCl.

Subsequent treatment of the gels is described in Section II 2B (ii). Where applicable, stained gels were scanned in a Gilford linear transport gel scanner at 615 nm.

(b) SDS-containing polyacrylamide slab gels with discontinuous buffers

Two methods for separating denatured polypeptides have been utilised in this thesis. The first, based on the procedure of Laemmli (1970) uses discontinuous Tris buffers; the second, a modification of the Laemmli procedure (Chua and Bennoun, 1975) uses Tris and borate-based buffers.

In all cases, the slab gel apparatus used was a modification of that described by Studier (1973). The running plates were 20 x 20 cm sheets of glass, 0.3 cm in thickness. One of the pair of plates had a notch of glass removed from the upper edge (16 x 1.5 m) so that when it was placed against the upper reservoir, electrode buffer could flow freely into the gel slots. Gel thickness was determined by spacing strips placed along the vertical edges of the plates (20 x 1.0 x 0.15 cm). The plates were sealed around the spacing strips and bottom edge by a length of silicon rubber tubing (0.25 cm diameter) and held together by clips during formation of the gel. The clips and rubber tubing were removed before the plates were inserted into the electrophoresis tank, and the apparatus was made leak-proof with solid white paraffin.

Both Laemmli and Chua-type linear polyacrylamide gradient gels were cast as follows. The high percentage acrylamide gel mixture (20 ml) was pipetted into a universal bottle and stirred vigorously by two small fleas. One volume of low percentage acrylamide gel mixture from a 20 ml stock was pumped into the high percentage gel mixture and, at the same time, 2 volumes were pumped out of this mixture and into the gel plates. Pumping was continued until all the gel solutions (40 ml) had been poured into the plates. The surface of the gel was overlaid with 5 ml distilled

water and polymerisation allowed to proceed. Under the conditions described below, polymerisation was completed by about 30 minutes after pouring. The volume of gel mixture used (40 ml) gave a resolving gel about 15 cm in length.

When the resolving gel had polymerised, the water was poured off and the top of the gel washed with 2 ml stacking gel mixture. The stacking gel was then poured from a 20 ml stock mixture and a Teflon slot-former with either 12 places, each 1.8 x 1.0 cm, or 14 places, each 2.0 x 0.6 cm, placed in the top of the plates. When polymerisation of the stacking gel was complete, the slot-former, rubber tubing and clips were removed and the plates inserted into the electrophoresis tank. The upper and lower reservoirs were filled with the appropriate buffers, and the gel was then ready for loading.

Laemmli-type gels were cast as 10-30% (w/v) linear polyacrylamide gradients as described above. The 30% gel mixture contained 30% (w/v) acrylamide, 0.15% (w/v) bisacrylamide, and the 10% gel mixture contained 10% (w/v) acrylamide, 0.27% (w/v) bisacrylamide from acrylamide stock solutions of 60% (w/v) acrylamide, (0.3% (w/v) bisacrylamide and 30% (w/v) acrylamide), 0.8% (w/v) bisacrylamide respectively. Both mixtures also contained final concentrations of 375 mM Tris-HCl (pH 8.8), 0.2% (w/v) linear polyacrylamide (M wt. greater than 10^6) to stabilise the gradient during pouring, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate and 0.03% (v/v) TEMED to effect polymerisation.

The stacking gel in this system contained 6% (w/v) acrylamide, 0.16% (w/v) bisacrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.125% (v/v) TEMED. The length of the stacking gel from the top of the resolving gel to the bottom of the slots was normally 2-3 cm. The electrophoresis buffer used in both the upper (cathode) and lower (anode) reservoirs

contained 25 mM Tris-HCl (pH 8.5), 0.192 M glycine and 0.1% (w/v) SDS. Gradient gels (10-30%) were normally electrophoresed for 12 hours at 20 mA constant current. After this time, the bromophenol blue dye marker had run off the bottom of the resolving gel.

Chua-type gels were cast either as 10-30% (w/v) or7.5-15% (w/v) linear polyacrylamide gradients as described above. The 10% gel mixture contained 10% (w/v) acrylamide, 0.27% (w/v) bis-acrylamide, the 15% gel mixture contained 15% (w/v) acrylamide, 0.4% (w/v) bisacrylamide, and the 30% gel mixture contained 30% (w/v) acrylamide, 0.15% (w/v) bisacrylamide. All the gel mixtures contained 0.424 Tris-HCl (pH 9.2), 0.1% (w/v) SDS, 0.2% (w/v) linear polyacrylamide, 0.03% (w/v) ammonium persulphate and 0.03% (v/v) TEMED. In this system, the stacking gel contained 6% (w/v) acrylamide, 0.16% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.125% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. The stacking gel buffer was a Tris-H_2SO_4 buffer, containing 0.054 M Tris-H_2SO_4 (pH 6.1). Neville upper reservoir buffer (0.04 M boric acid, 0.041 M Tris, 0.1% (w/v) SDS. pH 8.7) was used in the upper (cathode) reservoir whilst resolving gel buffer was used in the lower (anode) reservoir to the same final concentration in the gel. The effect of these buffer modifications is to improve stacking of the sample as it moves from one buffer zone to the next, and it also allows large volumes (up to 200 µl) of sample to be loaded on to each slot.

After electrophoresis, the gel was removed from the plates on to a plastic grid and transferred to a stain solution containing 0.25% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol, 7% (v/v) acetic acid for 2 hours. Destaining was carried out in 40% (v/v) methanol, 7% (v/v) acetic acid over a period of 24 hours with at least four changes of destaining solution.

(ii) RNA gels

Samples of chloroplast RNA were analysed by electrophoresis on cylindrical gels containing 2.4% (w/v) acrylamide as described by Loening (1967). To remove impurities, the acrylamide was recrystallised from chloroform and the bisacrylamide recrystallised from acetone.

Cylindrical gels, 9 cm in length, were cast in Perspex tubes (0.6 x 10 cm) sealed at one end with silicon rubber and a glass plug. Each gel contained 2.4% (w/v) acrylamide, 0.12% (w/v) bisacrylamide, 36 mM Tris, 30 mM NaH_2PO_4 and 1 mM EDTA. The gel mixtures were degassed under vacuum, and polymerisation started by the addition of TEMED (0.1%, v/v) and ammonium persulphate (0.2%, w/v). The gel mixture was poured into the tubes, overlaid with water, and polymerisation completed. The glass plugs were removed and the upper (cathode) and lower (anode) tank filled with electrophoresis buffer containing 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA and 0.2% (w/v) SDS (Loening, 1969). The gels were electrophoresed for 30 minutes at 6 mA/gel at room temperature.

The chloroplast RNA samples were dissolved in buffer containing 18 mM Tris, 15 mM NaH_2PO_4, 0.5 mM EDTA, 5% (w/v) sucrose, 0.1% (w/v) SDS and 0.05% (w/v) bromophenol blue. The samples were loaded on to the gels and electrophoresed for 3 hours at 6 mA/gel at room temperature. After this time, the bromophenol blue dye had just started to leave the bottom of the gel. The gels were removed from the tubes, soaked for 30 minutes in distilled water and scanned in a Gilford gel scanner at 260 nm.

G. Peptide mapping by partial proteolytic digestion

In order to confirm the identity of polypeptides synthesized in a rabbit reticulocyte cell-free system programmed with chloroplast RNA, the electrophoresed products were subjected to partial

proteolytic digesion mapping as described by Cleveland et al. (1977). The technique is especially convenient because it is more rapid than conventional tryptic fingerprint analysis and it requires only the small amounts of protein contained in a single gel band. The partial digests produced are relatively stable and contain a pattern of peptides which is characteristic of the protein substrate and proteolytic enzyme chosen.

Cell-free system products (Section II 2E (ii)b) and products of protein synthesis in isolated chloroplasts incubated with [^{35}S]-methionine (Section II 2D (i)) were electrophoresed on a 10-30% linear polyacrylamide gradient Laemmli gel containing SDS (Section II 2F (i)b). The gel was cast with spacing strips 1 mm thick and 14 slots, each 2.0 x 0.6 cm. The gel was electrophoresed for 12 hours at 20 mA (constant current) as previously described, removed from the plates, and then stained for 30 minutes in 0.1% (w/v) Coomassie Brilliant Blue R250, 50% (v/v methanol, 10% (v/v) acetic acid. Destaining was carried out in 5% (v/v) methanol, 10% (v/v) acetic acid for 1 hour, during which time the gel expanded to almost twice the original size. After a brief rinse in cold, distilled water,the gel was transferred to a glass plate, illuminated from beneath. Regions of the gel corresponding to the large subunit of Fraction I protein and Peak D were excised, using a Corrux blade,and soaked for 30 minutes with occasional swirling,in 10 ml sample buffer (125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 1 mM EDTA).

A second gel (Laemmli type) was cast having 1.5 mm spacing strips and 12 slots each 2.0 x 1.0 cm. The stacking gel was 5 cm in length and the gel solutions contained 1 mM EDTA (final concentration). The resolving gel contained 15% (w/v) acrylamide (Section II 2F (i)b). Presoaked gel slices were pushed to the bottom of the new wells with a spatula and each slice overlaid with

10 µl 20% (v/v) glycerol in sample buffer. Finally. 10 µl 10% (v/v) glycerol in sample buffer containing 0.5 µg Staphylococcus aureus V8 protease was layered over each slice. This protease cleaves at the carboxy-terminal side of aspartate and glutamate residues (Houmard and Drapeau, 1972). Electrophoresis was carried out as described above, except that the current was switched off for 30 minutes when the marker dye had nearly reached the end of the stacking gel. This gel was prepared for fluorography, dried down and autofluorographed as described in Section II 2K.

H. Rehydration and solubilisation of dried-down slab gels for scintillation counting

The method used was developed in this laboratory (Barraclough and Ellis, 1978) for rehydration of gels dried down on to Whatman 3 MM paper (Section II 2K).

The gel and backing paper were immersed in 1 litre 40% (v/v) methanol, 7% (v/v) acetic acid until the gel could be peeled away from the paper (about 10 minutes). As the gel took up the destaining solution, it began to shrink until it was smaller than the dried-down size. The freed gel was transferred to distilled water for 15 minutes which allowed it to swell up to almost dried-down size. The gel was then transferred to a glass plate and the required bands chopped out. The bands were cut out to the same size, and two background slices cut out of blank gel. Each piece of gel (0.4 cm wide and less than 1 cm in length) was transferred to the bottom of a plastic vial insert, covered with 0.2 ml hydrogen peroxide solution (100 vol) and capped. The gel slices were digested at 50° C for 16 hours and any traces of gel remaining removed by raising the temperature to 80° C for 1 hour. Samples were cooled and 4 ml Triton/toluene scintillation fluid (0.4% (w/v) PPO, 0.05% (w/v) POPOP in toluene-Triton X-100 (2:1, v/v)) added.

Samples were shaken until clear and their radioactivity measured in a Packard Tricarb liquid scintillation spectrometer at 12% gain for 10 minutes. Each tube was counted at least twice to check for fluorescence effects.

I. Protein determinations

Protein content of samples was measured by the method of Lowry et al. (1951). The following standards were made up.

Solution A: 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) potassium sodium tartrate.

Solution B: 50 ml 2% (w/v) Na_2CO_3 mixed with 1 ml solution A, just before use.

Solution C: 1 M Folin-Ciocalteau reagent, diluted from a 2.5 M stock solution with distilled water.

A standard curve of 0-150 µg of protein was prepared from a stock solution of 1 mg/ml BSA in 1 M NaOH, and each tube made up to 0.5 ml by addition of 1 M NaOH. Test samples were precipitated by the addition of a final concentration of 5% (w/v) trichloroacetic acid and incubation for 1 hour on ice. The samples were boiled 15 minutes, cooled, and the precipitates collected by centrifugation at 2500 g for 10 minutes. The pellets were washed once in 10 ml 80% (v/v) acetone to remove lipid and other organic materials which may interfere with the colorimetric reactions, dried and dissolved in 0.5 ml 1 M NaOH.

All tubes were incubated for 30 minutes at 90° C, cooled, and 5 ml Solution B added to each. After mixing, 0.5 ml Solution C was added, the tube contents remixed and incubated 30 minutes at room temperature. A_{750} nm was measured in a Unicam SP 500 spectrophotometer against the blank, and the protein concentration in the test samples determined from the standard curve.

J. Estimation of chlorophyll

Chlorophyll concentrations were determined by the method of Arnon (1949). Aliquots of leaf homogenate of chloroplast suspension were adjusted to a final concentration of 80% (v/v) acetone and incubated in the dark at room temperature for 10 minutes. Precipitated material was removed by either a low speed centrifugation in the case of small volumes, or by filtration through Whatman No. 1 paper. The absorption at 645 nm and 663 nm was read in a Unicam SP 500 spectrophotometer against a blank of 80% (v/v) acetone.

Chlorophyll concentration was calculated from the following formula:-

$$\mu g/ml \text{ chlorophyll in the acetone extract} = (20.2 \times A_{645}) + (8.02 \times A_{663})$$

K. Fluorography and autoradiography

Gels to be autoradiographed were dried down by a modification of the method of Maizels (1971). The wet, fixed gel was transferred on to a sheet of Whatman 3 MM filter paper with a clear margin of 2 cm around the gel. The papers was placed on the drying apparatus and the gel covered with a single sheet of heavy-duty clingfilm, followed by a piece of card 2 mm thick. The apparatus was then enclosed in a Sterilin bag, held closed by 4 clips. The drier consisted of a metal frame holding a piece of porous polyethylene on to which the filter paper was placed. Water from the gel was sucked through the porous sheet and out of the back of the plate by a vacuum line. Drying was normally carried out for 90 minutes at 80° C, after which the gel was autoradiographed against Kodirex film (18 x 24 cm).

Where gels were to be fluorographed by the method of Bonner and Laskey (1974), the gel was impregnated with PPO prior to drying down by the above method. The film (Cronex 4) was sensitised by preflashing (Laskey and Mills, 1975) for 2 seconds with white light. The light source was a darkroom enlarger with the lens set at f22 and covered with one sheet of Whatman 3 MM paper. The film was 94 cm from the lens. Exposure of the film to the gel was carried out at -90° C.

L. Extraction of protein from non-denaturing gels

In order to examine the purity of the Fraction I protein bands resolved on 6% non-denaturing gels (Section II 2F(i)a), the bands were excised, the protein extracted and re-electrophoresed on an SDS polyacrylamide gel. Unstained bands of Fraction I protein were identified by alignment with a stained marker gel, then excised with a razor blade. The excised bands from 6 gels were homogenized by passing through a 5 ml syringe (without a needle) and the homogenate passed into 2 ml extraction buffer (2.5 mM Tris, 19 mM glycine, 2% (w/v) SDS, 100 mM 2-mercaptoethanol (pH 8.5)). The slurry was boiled for 2 minutes and then stirred for 2 hours at room temperature. The gel fragments were filtered out with cotton-wool and re-extracted with a further 1 ml extraction buffer. The two eluates were pooled and refiltered through cotton-wool to remove traces of gel. After dialysis against 2.5 mM Tris-glycine buffer (pH 8.5), 0.1% (w/v) SDS, 10 mM 2-mercaptoethanol overnight, the SDS-protein extract was electrophoresed on a 10-30% polyacrylamide gradient Chua gel (Section II 2F(i)b) for 15 hours at 20 mA constant current.

M. Preparation of an enriched chloroplast coupling factor fraction

A fraction enriched in CF_1 polypeptides was prepared by the method of Strotmann et al. (1976). Washed chloroplasts prepared from 15 g primary leaves (Section II 2D(i)) were resuspended in 20 ml of a solution containing 10 mM sodium pyrophosphate-HCl (pH 7.5) and centrifuged at 30 000 g for 10 minutes at 0-4° C. The supernatant liquid was discarded and the pellet washed twice more in 20 ml sodium pyrophosphate solution, and centrifuged each time at 30 000 g for 10 minutes at 0-4° C. The final pellet was divided into two portions. One portion was resuspended in 3 ml of a buffer containing 0.3 M sucrose, 2 mM Tricine-KOH (pH 7.5) and centrifuged at 135 000 g for 30 minutes at 0-4° C. The supernatant fraction, which contains CF_1 polypeptides, was decanted using a Pasteur pipette, and retained on ice. The pellet was re-extracted with a further 3 ml sucrose-Tricine buffer, and the two supernatant fractions pooled. The pooled supernatants were made 2% (w/v) in SDS, 100 mM in 2-mercaptoethanol and boiled for 2 minutes.

The second portion of sodium pyrophosphate-washed pellet was resuspended in 6 ml of 2.5 mM Tris-19 mM glycine buffer (pH 7.5), 2% (w/v) SDS, 100 mM 2-mercaptoethanol and boiled for 2 minutes.

The fractions were analyzed on Chua SDS gels (Section II 2F(i)b) containing a 7.5-15% polyacrylamide gradient, and the polypeptides visualised by staining with Coomassie Blue dye.

SECTION III - RESULTS AND DISCUSSION

1. CHARACTERISTICS OF DEVELOPMENT OF THE PRIMARY LEAF PAIR IN SPINACH

Since the key question in this thesis is whether the products of chloroplast protein synthesis change qualitatively or quantitatively during chloroplast development, it is important to have some measure of the physical and biochemical changes taking place in the whole leaf. These data serve two purposes:-

(1) they define the time-limits of synthesis of chloroplast components within the leaf, and indicate when these components cease to accumulate;

(2) since two different sets of growth conditions have been used, the parameters of growth may be used to express subsequent results from the two types of plant on a common basis.

A. Measurement of physical leaf parameters.

(i) Fresh and dry leaf weights

Figure 1 illustrates the changes in leaf fresh and dry weight during the growth of the primary leaves of compost-grown plants. Although fresh weight increases throughout the whole period measured, the maximum rate of increase occurs between days 18 and 26, when the doubling time is 4 days. In contrast, dry weight does not parallel the dramatic increase in fresh weight but increases at a low linear rate, doubling between days 22 and 40. At day 20, the dry matter accounts for about 65% of the leaf fresh weight, but by day 36 this has decreased to about 9%. This change indicates that in the later stages of leaf expansion, most of the increase in weight is due to water uptake.

Figure 1 also compares the fresh weights of compost-grown leaves with those of hydroponically-grown leaves. It is apparent that hydroponically-grown leaves grow faster, and reach a higher

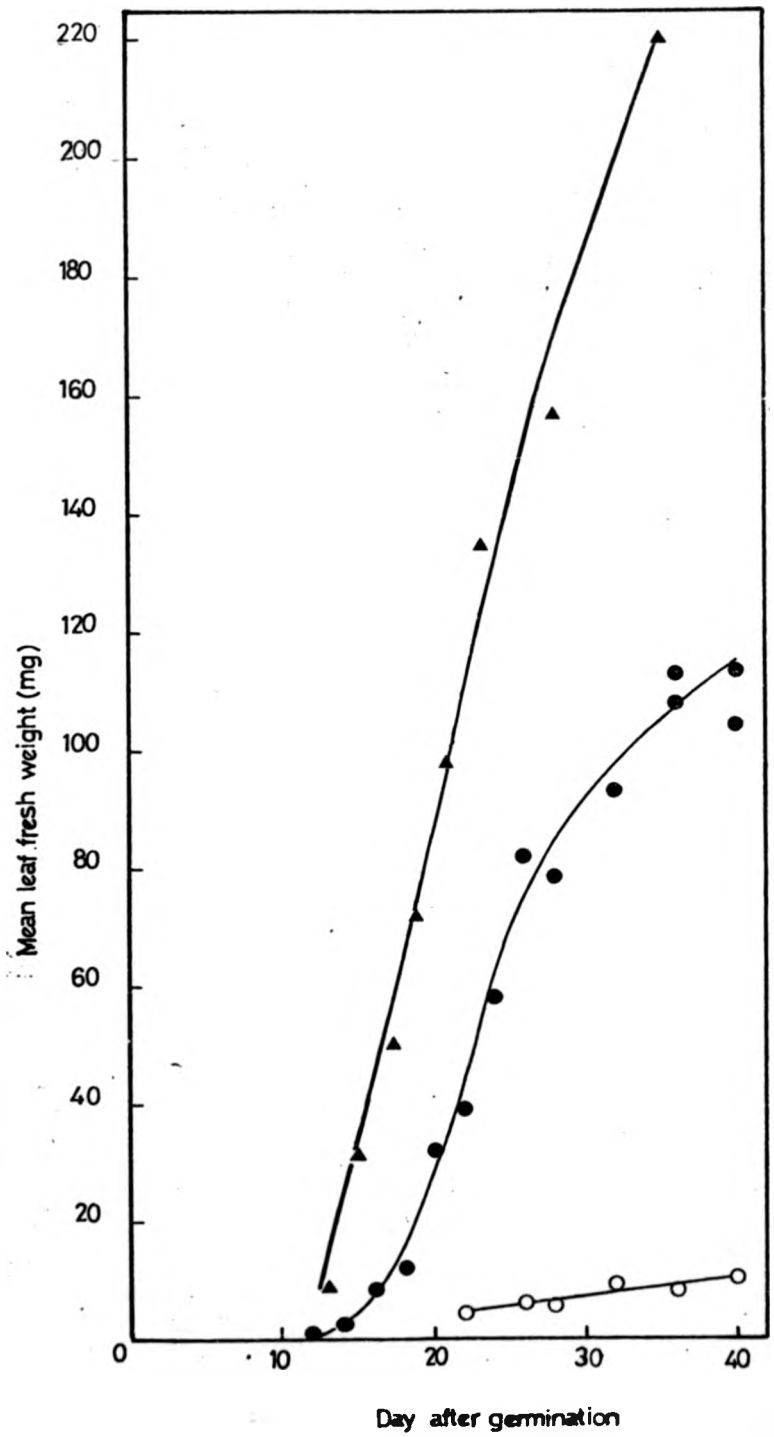

220
200
180
160
140
120
100
80
60
40
20
0
10
20
30
40
Mean leaf fresh weight (mg)
Day after germination

Figure 1

Changes in spinach primary leaf fresh weight and dry weight during growth.

Individual primary leaves were detached at the leaf base at the same time on each day and immediately weighed on a four-figure balance. Each point is the mean of two samples, each containing at least 10 leaves.

For dry weight estimation, 5 samples of 5 leaves were placed in a Unicam vial (preweighed and capped) which had previously been dried for 16 hours at 90° C and then cooled before use. The leaf samples were dried at 90° C for at least 24 hours and weighed at intervals until the dry weight remained constant. The bottles were capped and cooled before each reweighing.

●——● mean fresh weight per compost-grown leaf

○——○ mean dry weight per compost-grown leaf

△——△ mean fresh weight per hydroponically-grown leaf

value of fresh weight than do compost-grown leaves. At day 14, when the compost-grown leaves are increasing in fresh weight at a low rate, the hydroponically-grown leaves are already increasing at a high linear rate.

(ii) Leaf length

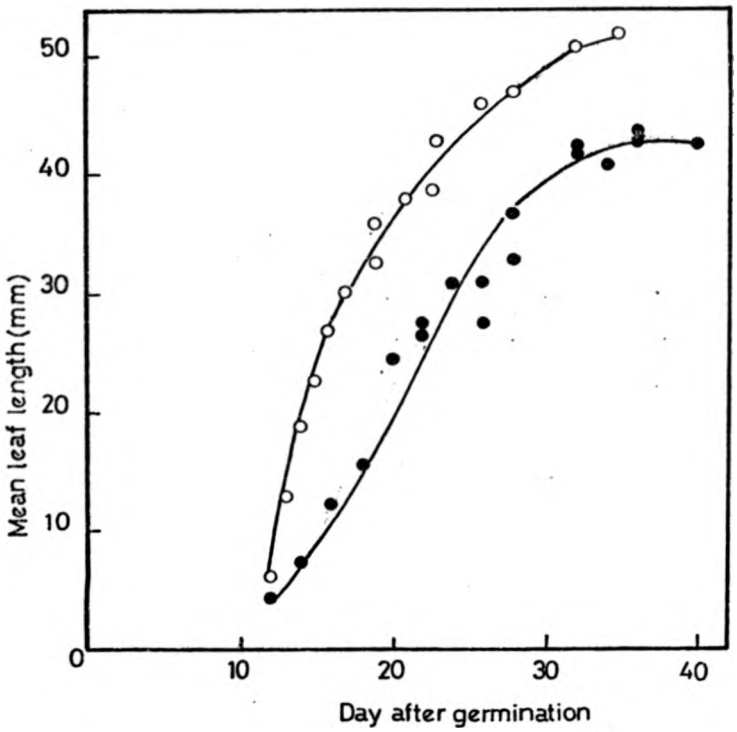
The change in leaf length during the growth of compost- and hydroponically-grown leaves is shown in Figure 2a. The difference in growth pattern for the two types of leaf becomes apparent at the earliest points measured, when the leaves are about 5 mm in length. In hydroponically-grown leaves, the rate of increase in leaf length is linear from this time until day 16 when the rate falls off gradually. This pattern contrasts with the growth pattern of compost-grown leaves where linear growth continues until day 28, although at a lower rate than in liquid culture. Unlike the hydroponically-grown leaves, compost-grown leaves stop increasing in length soon after the linear rate of increase in length is over. The hydroponically-grown leaves, therefore, as a result of these factors, reach a final length which is greater than that for compost-grown leaves.

Despite the differences between the growth patterns of the two types of leaf, there is an identical relationship between leaf length and fresh weight in both cases (Fig. 2b). This relationship implies that although the rates of growth, final leaf length and fresh weight differ in the two types of plant, the leaves are comparable at any particular leaf length. That is, the leaves have a common pattern of regular growth.

(iii) Leaf area

There is a marked increase in leaf area in compost-grown leaves during their development. The data, shown in Figure 3, indicate that the maximum rate of increase in leaf area falls

(a)


Mean leaf length (mm)
0
10
20
30
40
50
10
20
30
40
Day after germination

(b)

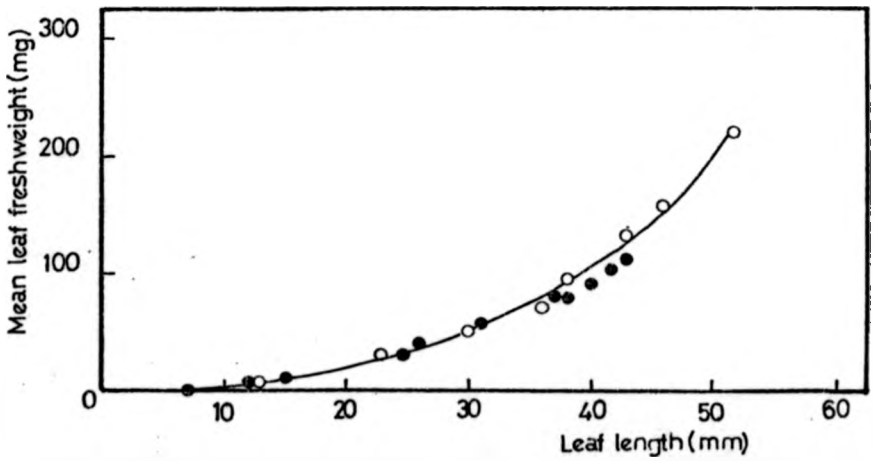

Mean leaf freshweight (mg)
0
100
200
300
10
20
30
40
50
60
Leaf length (mm)

Figure 2

Changes in spinach primary leaf length during growth.

Individual primary leaves were detached at the leaf base and the length from base to tip estimated by placing the leaf on graph paper ruled in mm. Each point is the mean of two samples each containing at least 10 leaves.

(a) Mean leaf length *versus* day after germination

(b) Mean leaf fresh weight (from Fig. 1) *versus* leaf length

●——● compost grown leaves

○——○ hydroponically-grown leaves

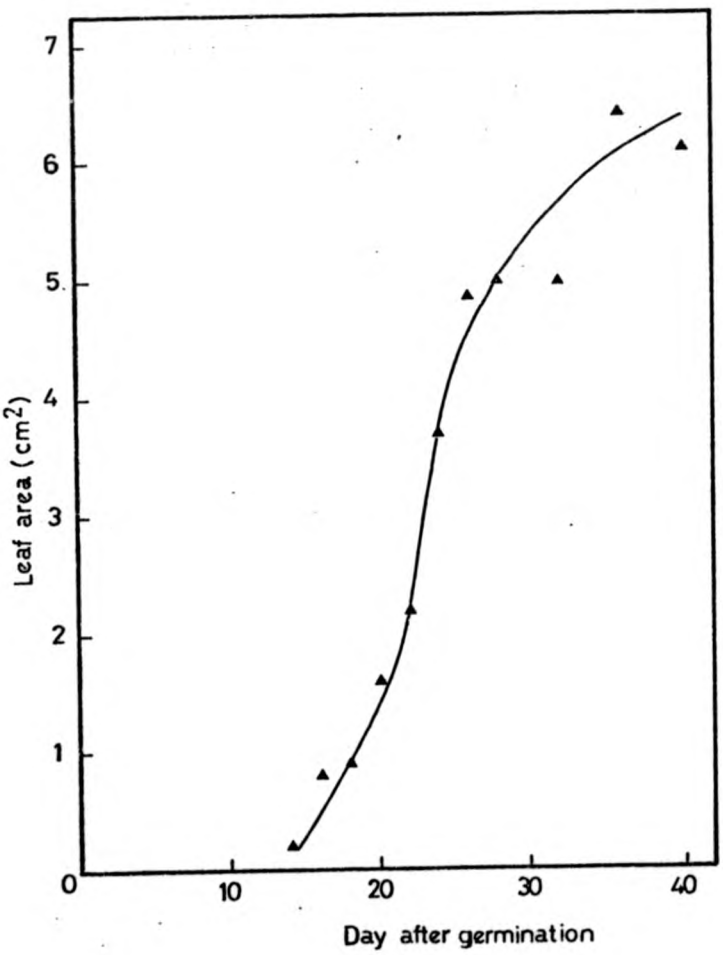

Leaf area (cm^2)
0
1
2
3
4
5
6
7
10
20
30
40
Day after germination

Figure 3

Changes in leaf area during spinach primary leaf growth.

Primary leaves were detached from compost-grown plants at the leaf base and placed on graph paper ruled in mm. The leaf outline was drawn and this outline cut out and weighed. A standard curve (not shown) was constructed by weighing known areas of graph paper. Each point is the mean of ten leaves, whose weight was used to estimate area from the calibration curve.

between days 20 and 28, a shorter time period than for the maximum rate of increase in fresh weight. This difference implies that the primary leaves are not simply expanding over their entire area during growth,since there are times when leaf area increases at a slower rate than leaf length. When leaf length is compared directly with leaf area (Fig. 4a), the relationship is seen to be biphasic. When the leaves are smaller than 25 mm in length, leaf length increases at a faster rate than leaf area; when the leaves are longer than 25 mm, the converse is true. This relationship is not found between leaf area and leaf fresh weight (Fig. 4b). The linear relationship observed between leaf area and leaf fresh weight throughout the entire growth period implies that leaf thickness is not a major contributor to the increase in fresh weight which occurs during leaf expansion.

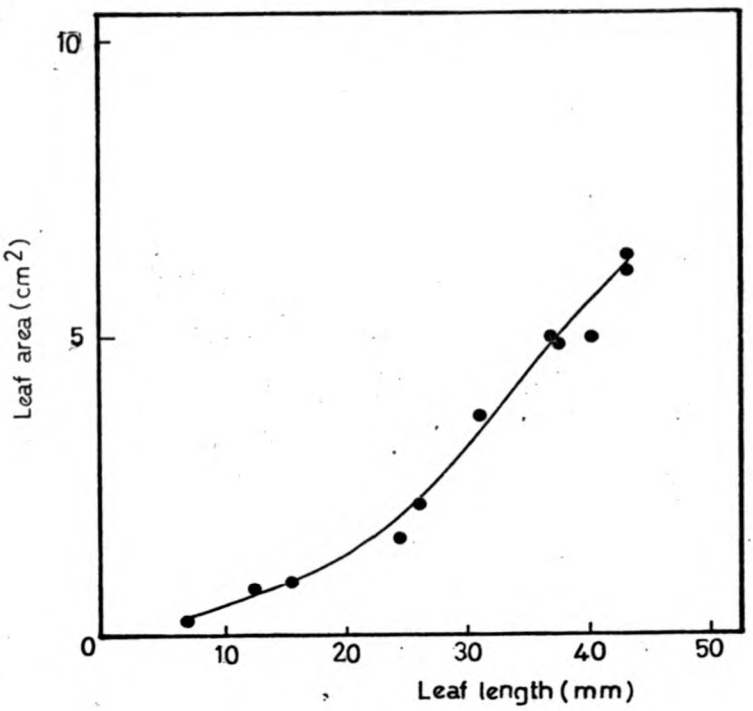
The data in Figures 1-4 imply that as leaves increase in length there is expansion over the whole leaf area. Moreover, the increase in fresh weight during leaf growth shown in Figure 1 is not due to increasing leaf thickness, but rather to the increase in leaf area. This dramatic increase in leaf area during growth can be seen clearly in Figure 5, which shows compost-grown primary leaves 17 and 45 days after germination.

B. Biochemical leaf parameters

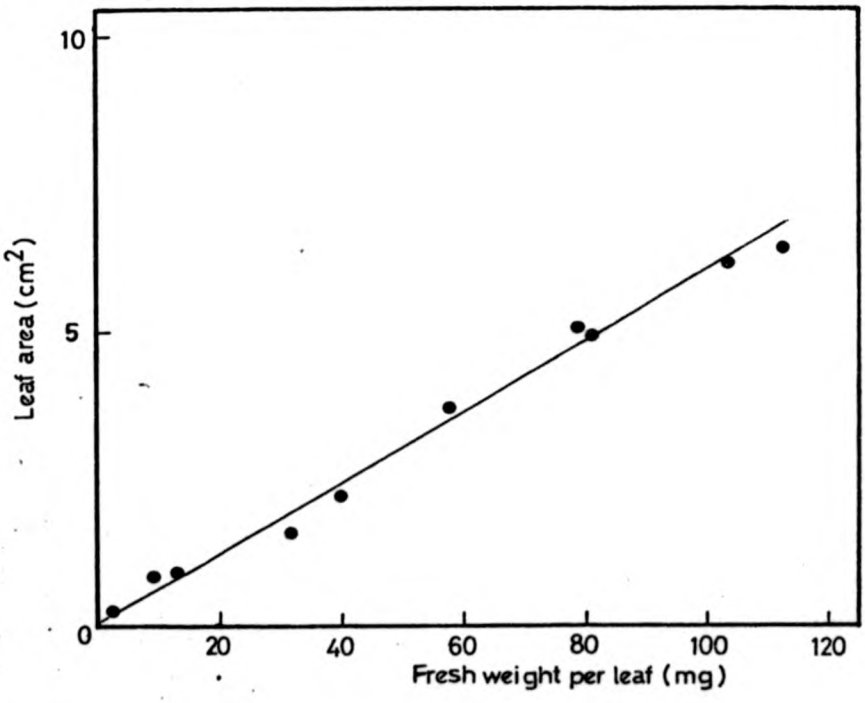
(i) Leaf chlorophyll content

In this section, three of the biochemical parameters of leaf development which reflect plastid development were measured. The change in chlorophyll content with age for hydroponically- and compost-grown primary leaves is shown in Figure 6a. The compost-grown plants maintain a maximum rate of accumulation of chlorophyll in the primary leaves between days 18 and 30, whereas hydroponically-grown plants show a faster increase but slow down earlier, at day 26.

(a)


10
5
0
Leaf area (cm²)
10
20
30
40
50
Leaf length (mm)

(b)


10
5
0
Leaf area (cm²)
20
40
60
80
100
120
Fresh weight per leaf (mg)

A B

Figure 4

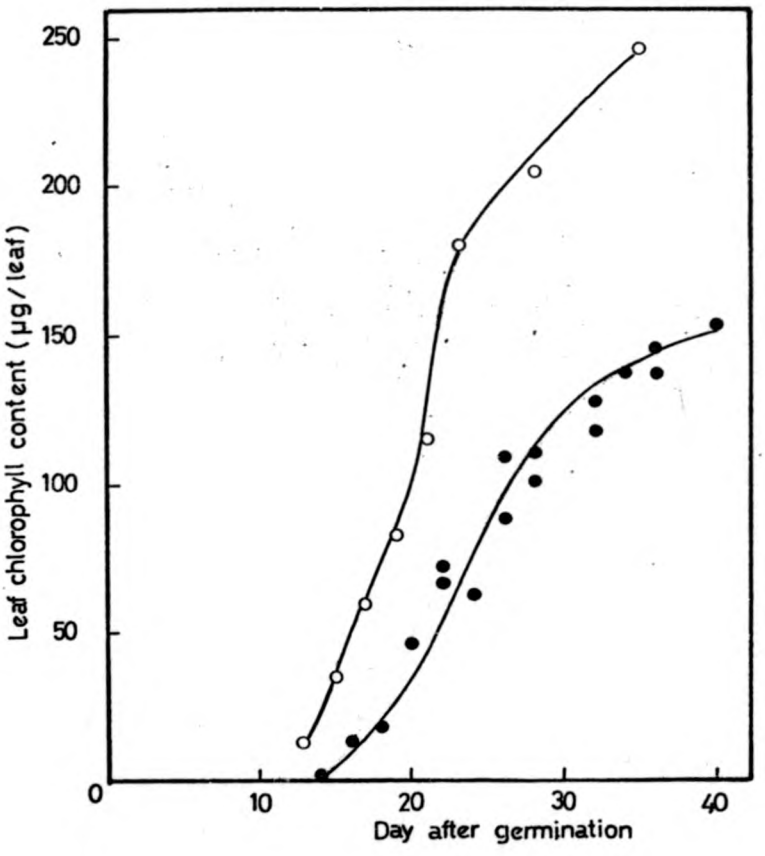
Leaf area as a function of other leaf parameters.

Leaf areas from Figure 3 were plotted against corresponding values for compost-grown leaves.

(a) Leaf length (from Figure 2)

(b) Fresh weight (from Figure 1)

(a)


250
200
150
100
50
0
Leaf chlorophyll content (µg/leaf)
10
20
30
40
Day after germination

(b)

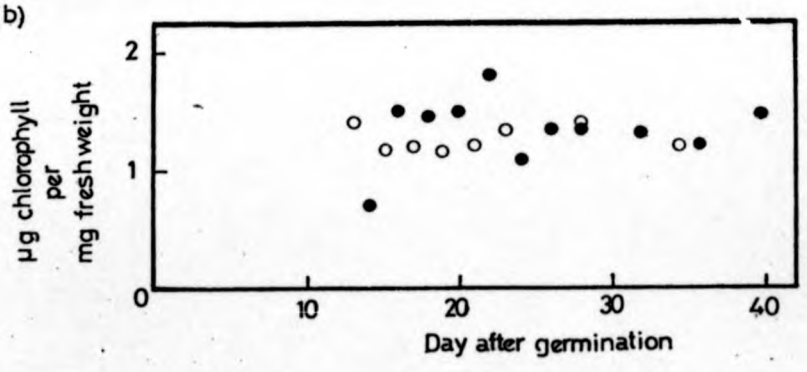

2
1
0
µg chlorophyll per mg fresh weight
10
20
30
40
Day after germination

Figure 5

Comparison of young and old spinach plants.

Compost-grown plants were removed from the soil and photographed at:-

A, 17 days after germination

B, 45 days after germination.

The primary leaf pair is arrowed on each plant.

Figure 6

Changes in spinach primary leaf chorophyll content during growth.

Leaf homogenates were prepared by the method described in Section II 2A and chlorophyll measured in aliquots (Section II 2J). The values plotted are means of two samples each made from at least 10 leaves. In the compost-grown leaf data, two separate batches of plants contribute points.

(a) Chlorophyll per leaf *versus* time

(b) Chlorophyll on a fresh weight basis *versus* time

O——O hydroponically-grown plants

●——● compost-grown plants

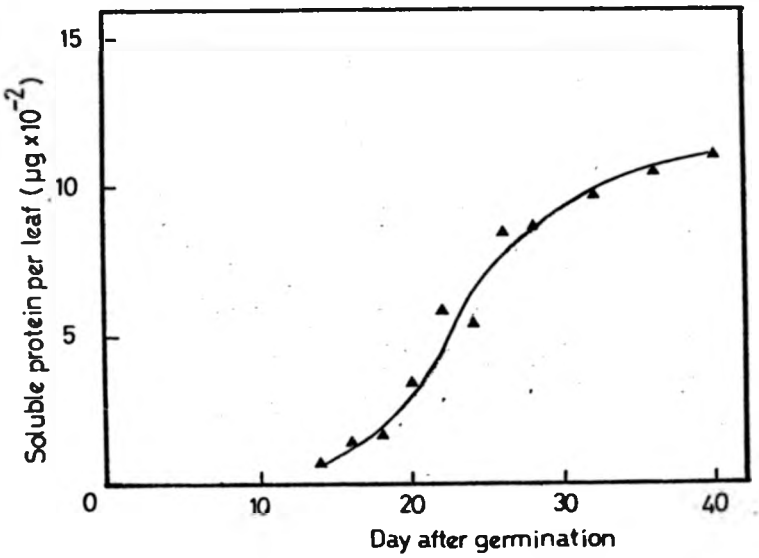
This pattern of chlorophyll accumulation in the two types of leaf reflects the increase in leaf fresh weight during development. Thus, although the hydroponically-grown plants accumulate more chlorophyll per leaf than the compost-grown plants, chlorophyll content on a fresh weight basis is similar in the two types of plant throughout development (Fig. 6b).

(ii) Leaf soluble protein

The pattern of accumulation of soluble protein during primary leaf development is shown in Figure 7. The term "soluble" here is an operational definition, indicating that protein which remains in the supernatant liquid at the end of the leaf fractionation procedure described in Section II 2A. This protein fraction may also include loosely-bound membrane components removed by the buffer employed, components which may be insoluble *in vivo*. The soluble fraction will contain stromal proteins including Fraction I protein (Section I 2A).

The maximum rate of accumulation of soluble protein on a leaf basis takes place between days 18 and 28 in compost-grown primary leaves (Fig. 7a), a pattern closely following that for chlorophyll accumulation (Fig. 6a). This similarity is expected, since chloroplast protein accounts for up to 75% of total leaf protein (Stocking and Ongun, 1962), and thus the soluble protein content as well as the chlorophyll content reflects the increase in chloroplast numbers during leaf expansion. It is interesting that while soluble protein accumulates on a leaf basis throughout development, it decreases in the early stages of development when expressed in terms of fresh weight (Fig. 7b). After day 20, the increase in soluble protein per leaf keeps pace with increasing fresh weight. This changing pattern of soluble protein content on a fresh weight basis contrasts with the constant ratio of chlorophyll content expressed on the same basis (*cf.* Fig. 7b with Fig. 6b).

(a)


15
10
5
0
10
20
30
40
Soluble protein per leaf ($\mu g \times 10^{-2}$)
Day after germination

(b)

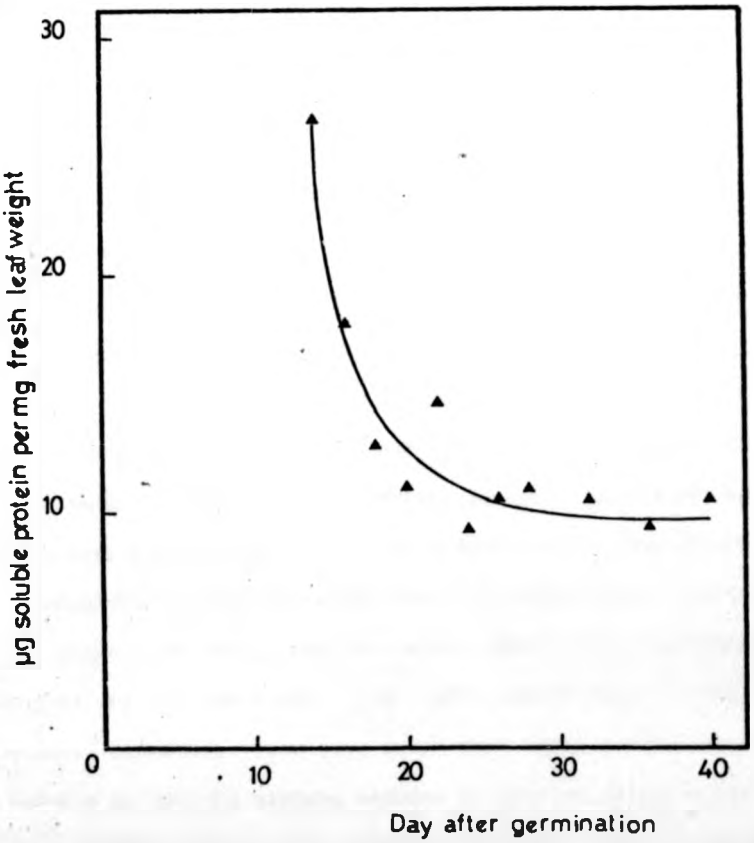

30
20
10
0
10
20
30
40
µg soluble protein per mg fresh leaf weight
Day after germination

Figure 7

Changes in soluble protein content during spinach primary leaf development.

A soluble protein fraction was prepared as described in Section II 2A, and assayed for protein content as described in Section II 2I. Points are means of two samples, each made from more than 10 leaves from compost-grown plants.

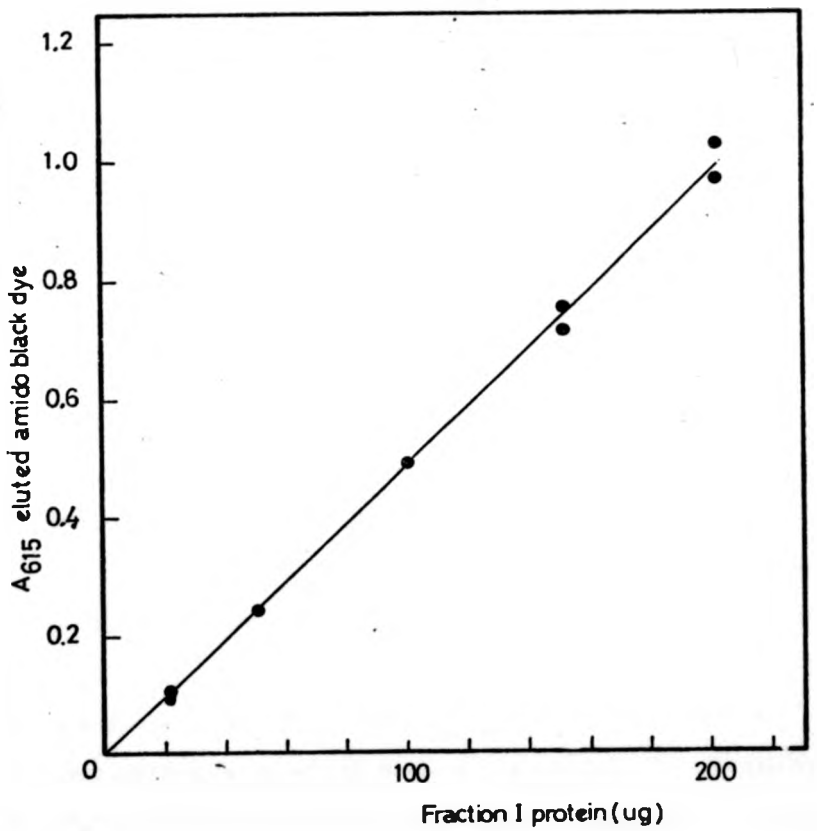
(a) soluble protein per leaf *versus* time

(b) ratio of µg soluble protein per mg fresh weight *versus* time

(iii) Fraction I protein

Fraction I protein is a major chloroplast protein both in amount and function, and its synthesis known to involve the protein-synthesizing system located in the chloroplast (Section I 2C). Measurements of this protein should therefore give an indication of chloroplast synthesis during leaf growth. The amount of Fraction I protein per leaf during leaf development was estimated from standard curves constructed with data from purified spinach Fraction I protein (Section II 2B(i) and (ii)). Under the standard conditions described, a reproducible calibration curve was obtained (Fig. 8). The identity of the band in soluble protein samples which co-electrophoresed with the native Fraction I protein marker was checked by eluting the band into an SDS buffer and re-electrophoresing the extract on an SDS-polyacrylamide gel. The results of this check are shown in Figure 9. The sample bands co-electrophorese with the large and small subunits of the Fraction I protein marker. The results shown in Figure 9 indicate that there is no detectable contamination of the Fraction I protein band on non-denaturing polyacrylamide gels with proteins of different molecular weight since no other bands are visible on the SDS gel.

A possible major source of loss of Fraction I protein during preparation of leaf soluble protein is the final pellet (Section II 2A). This loss may be due to unbroken cells or supernatant liquid trapped in the interstices of the insoluble material. Microscopic examination revealed few unbroken cells. Figure 10 shows the results of analysis of soluble and pellet fractions on non-denaturing polyacrylamide gels. As expected, the supernatant fractions (Fig. 10, band D) contain a major band which co-electrophoreses with the Fraction I protein marker (Fig. 10A). The pellet


1.2
1.0
0.8
0.6
0.4
0.2
0
100
200
A615 eluted amido black dye
Fraction I protein (ug)


LSU
SSU
A
B

Figure 9

Re-electrophoresis in SDS of native Fraction I protein from non-denaturing polyacrylamide gels.

Fraction I protein bands (unstained) were excised from 6% non-denaturing polyacrylamide gels (Section II 2F(i)a) of a soluble leaf protein fraction (Section II 2A) by alignment with a stained marker gel. The protein in the gel pieces was extracted by the method set out in Section II 2L and re-electrophoresed on a 10-30% polyacrylamide gradient Chua gel containing SDS (Section II 2F(i)b). The polypeptide bands were visualised by staining with Coomassie Blue dye.

A. Purified spinach Fraction I protein

B. Eluted Fraction I protein

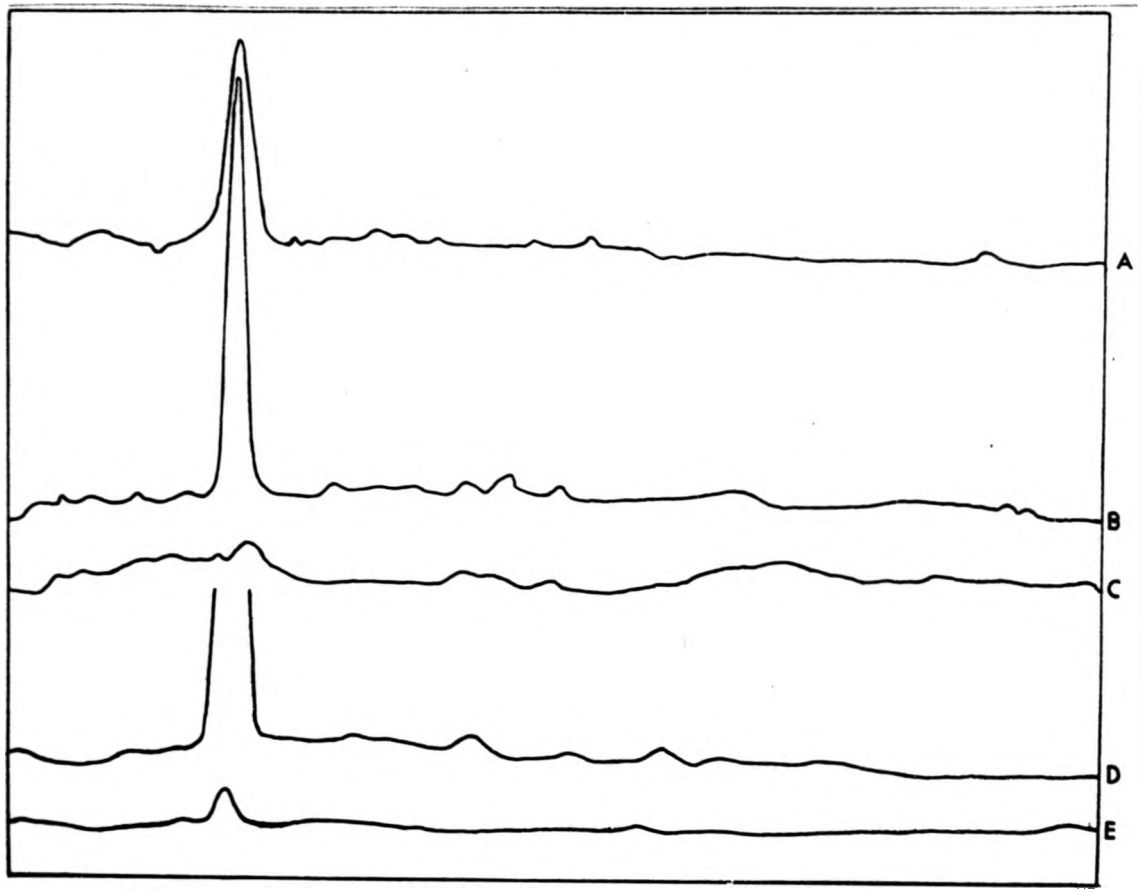

A
B
C
D
E

Figure 10

The distribution of Fraction I protein between soluble and insoluble protein fractions prepared from spinach primary leaves.

Spinach primary leaf material was fractionated into soluble and insoluble protein-containing supernatant and pellet fractions as described in Section II 2A. The final pellet was resuspended in a volume of grinding buffer equal to the supernatant volume. These native protein preparations were analysed on 6% non-denaturing polyacrylamide gels and the protein bands visualised with Amido Black dye. Panels show densitometer scans of the dye pattern:

A. Fraction I protein purified from spinach leaves

B. Supernatant protein

C. Pellet protein

D. Supernatant protein

E. Pellet protein

Tracks B and C were loaded with samples representing equal percentages of leaf extract. Tracks D and E were loaded with four times the sample loaded in B and C.

fractions (Fig.10 C and E) also contain a small amount of this protein, although the supernatant track had to be overloaded with respect to Fraction I protein before a band became visible in the corresponding pellet fraction. In the sample shown in Figure 10, the dye in the pellet band (E) represents 2% of the dye in the corresponding supernatant band (C). It is concluded that the pellet contains 2% of the Fraction I protein present in the supernatant fraction.

Figure 11 shows the increase in Fraction I protein per leaf that occurs during the development of both hydroponically- and compost-grown leaves. The hydroponically-grown leaves accumulate Fraction I protein at a faster initial rate than the compost-grown leaves, but the rate of accumulation slows down earlier. This pattern of behaviour is very similar to that found for the rate of accumulation of chlorophyll or fresh weight on a leaf basis, as discussed in Section III 1B(ii). In compost-grown plants, Fraction I protein becomes the major soluble leaf protein, accounting for about 80% by day 30 (Fig. 12a). In a period of 10 days, leaf Fraction I protein content increases 4-fold during maximal leaf expansion. This presumably mirrors the increase in chloroplast numbers when the spinach primary leaf is expanding at a maximum rate. The trend is also reflected in the pattern of bands seen on non-denaturing polyacrylamide gels of soluble protein fractions prepared from developing leaves (Fig. 12b). There are no major qualitative changes in the pattern of stained bands, but some quantitative changes. This observation partially reflects the insensitivity of the method of measurement and the predominance of Fraction I protein. A similar pattern of bands has been found in barley, pea and maize (Ellis, 1977).

In conclusion, the major changes taking place in the biochemical parameters measured are ones of accumulation.

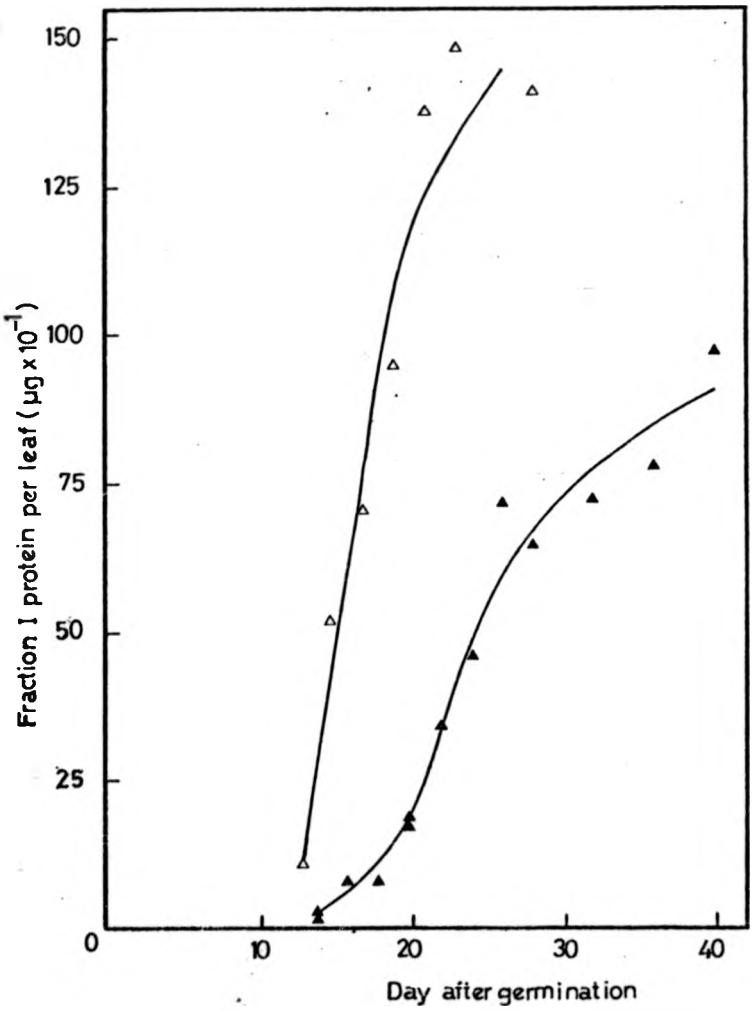

150
125
100
75
50
25
0
10
20
30
40
Fraction I protein per leaf (µg x 10⁻¹)
Day after germination

Figure 11

Changes in Fraction I protein per leaf during development.

Soluble protein fractions were prepared from leaves of increasing age by the method described in Section II 2A and analysed as described in Section II 2B(ii). Each point is the mean of two samples, each prepared from at least 10 leaves.

△——△ hydroponically-grown leaves

▲——▲ compost-grown leaves

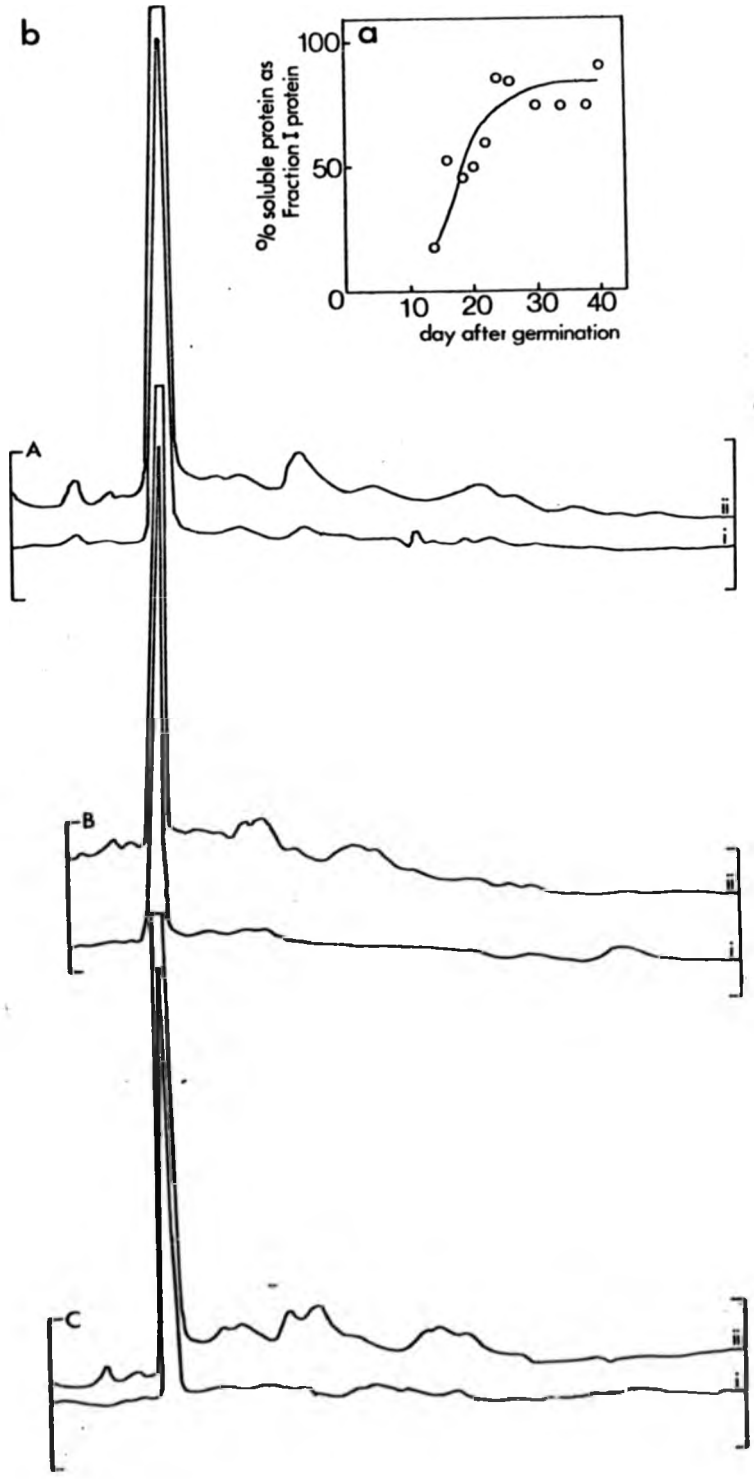

b
a
100
50
0
10
20
30
40
% soluble protein as Fraction I protein
day after germination
A
B
C

Figure 12

Changes in the amount of Fraction I protein relative to total soluble protein during development of spinach primary leaves.

(a) Fraction I protein measurements shown in Figure 11 are expressed as a percentage of the soluble protein values shown in Figure 7, for compost-grown leaves.

(b) Soluble protein fractions (Section II 2A) were analysed as described in Section II 2B(ii), and the dye-stained proteins scanned at 615 nm. The full scale is 2 absorbance units.

A. Soluble protein from day 22 leaves. Track (ii) contains twice as much protein as track (i).

B. Soluble protein from day 32 leaves. Track (ii) contains twice as much protein as track (i).

C. Soluble protein from day 36 leaves. Track (ii) contains 2.5 times as much leaf material as track (i).

C. Discussion

The structure of the mature chloroplast has been discussed in Section I 2A, and compared to etioplast structure in Section I 3. It was concluded that etiochloroplast development is not a tenable model for chloroplast development in many cases, so a study of leaf development in spinach grown under normal diurnal conditions of illumination was initiated. The results from this section indicate a pattern of growth which can be described and summarised as follows:-

(1) the way in which spinach plants are grown influences the course of their physical and biochemical development;

(2) during the course of spinach primary leaf development, leaf expansion is paralleled by an increase in the content of chlorophyll, soluble protein and Fraction I protein.

These points will now be discussed more fully.

The difference in growth pattern between hydroponically- and compost-grown plants is striking, and underlines the importance of environmental factors in plant growth (Humphries and Wheeler, 1963). Both types of plant were grown at a controlled temperature with the same photoperiod and light intensity at the plant level, so these factors are unlikely to be causes of the differences. The compost-grown plants were watered daily, so water-shortage is unlikely to be a factor in the smaller size and fresh weight attained by these leaves, as compared to their hydroponically-grown counterparts. The major difference between the two growth methods is that the roots of the hydroponically-grown plants are in aerated solution containing an ion balance optimised to the growth of spinach plants (Huntner, 1953). However, although at any particular time after germination the hydroponically-grown primary leaves are larger than the compost-grown leaves of the

same age, fresh weight or chlorophyll content for both is similar at any leaf length (Figs. 2b and 6b). Thus, while these plants exhibit different growth patterns, it is possible to express subsequent data on a comparable basis.

The pattern of growth shown by spinach primary leaves has also been found in a number of other species. In pea (Pisum sativum), chlorophyll content on both a leaf and fresh weight basis increases during leaf expansion (Smillie and Krotkov, 1961). Similarly, in cottonwood (Populus deltoides), the chlorophyll content of leaves increases during leaf expansion and there is also an increase in the carboxylase activity of Fraction I protein on both a chlorophyll and a fresh weight basis (Dickman, 1971). The amount of Fraction I protein in the first leaf of wheat (Triticum aestivum) increases during leaf expansion, reaching a maximum when leaf expansion has ceased (Patterson and Smillie, 1971) as in the spinach primary leaf. However, in a later study of the second leaf of wheat, Brady and Steele-Scott (1977) found that the amount of fraction I protein per leaf decreases after leaf expansion has ceased. These results are not necessarily incompatible since the more recent study is over a longer time period than that of Patterson and Smillie (32 days as opposed to 10 days), and during this time further leaves may have started to shade the leaf being sampled. This could have the effect of initiating the breakdown of Fraction I protein (Blenkinsop and Dale, 1974). Although these studies on wheat, pea and cottonwood are not directly comparable since different growth conditions were employed in each case, the general pattern of maximum accumulation of chlorophyll and Fraction I protein during leaf expansion was seen in each study.

In the present study, it is clear that the accumulation of Fraction I protein slows down during the later stages of leaf expansion (Fig. 11). This observation is consistent with evidence in barley (Hordeum vulgare) (Peterson et al., 1973), cottonwood (Dickman and Gordon, 1975) and Perilla frutescens (Woolhouse, 1967), where synthesis and turnover of Fraction I protein in mature leaves is negligible. In barley, it has also been shown that the soluble protein still turns over, even in nearly senescent leaves (Peterson et al., 1973). However, in the present study leaf length (Fig. 1), fresh weight (Fig. 2) and area (Fig. 3) in compost-grown leaves are constant between days 35 and 40 whilst Fraction I protein (Fig. 11), soluble protein (Fig. 7a) and chlorophyll (Fig. 6a) per leaf are still increasing. This suggests that chloroplast development proceeds for some time after leaf expansion has ceased.

In conclusion, the data presented in this section define the growth of spinach primary leaves under the set of conditions employed. These basic data form the essential basis for subsequent studies of chloroplast development. From the literature reviewed in Section I 3, it is clear that plastid replication takes place during leaf expansion in several species, including spinach (Possingham and Saurer, 1969; Honda et al., 1971). Thus, a knowledge of leaf expansion is important when choosing time points for the examination of changes in chloroplast protein synthesis.

III.2 IN VIVO LABELLING OF POLYPEPTIDES IN DEVELOPING SPINACH PRIMARY LEAVES

Physical and biochemical measurements of primary leaf development in spinach (Section III.1) indicate that the maximum rate of accumulation of both Fraction I protein and total leaf soluble proteins occurs during leaf expansion. The characterization of these developmental changes with respect to chloroplast protein synthesis in vivo will form the main part of this section. The aims of this section are as follows:-

1. to label and identify the products of chloroplast protein synthesis in developing leaves with [^{35}S]methionine;
2. to quantitate the changes in labelling of LSU relative to peak D during leaf development.

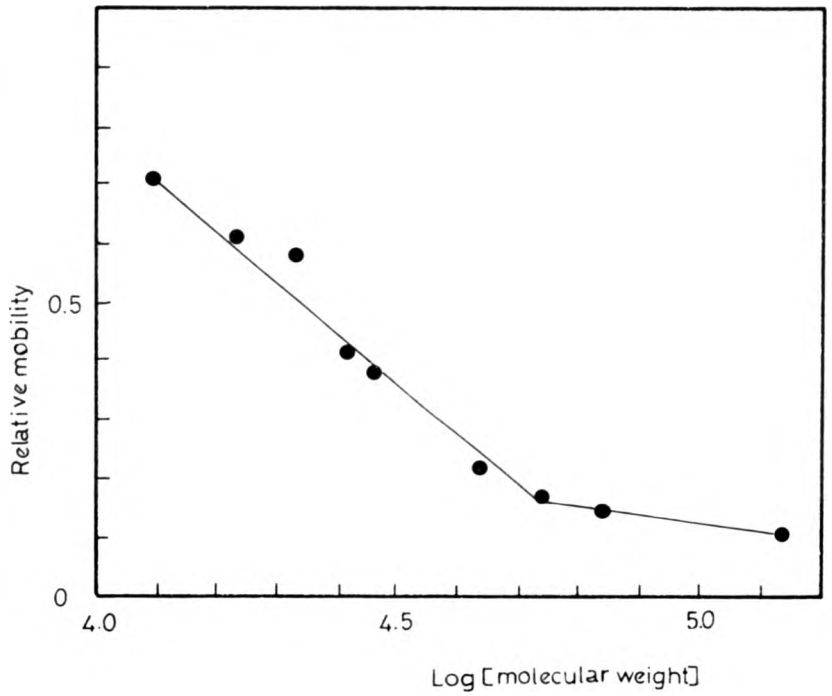
A. Electrophoretic analysis of in vivo-labelled polypeptides

The approach adopted for in vivo labelling was essentially that of Hartley and Ellis (1971). Spinach leaves were detached at the petiole base and a solution of isotope fed through the cut ends. This procedure has the advantage of allowing rapid labelling of polypeptides over a short time-period (6 hours), and avoids the problems of feeding whole plants through the roots. The radioisotope [^{35}S]methionine was chosen since it is available at high specific activities (up to 1000 Ci /mmol) compared to [^{14}C]-labelled amino acids (up to 300 mCi / mmol), and is readily detectable by autoradiography. Cashmore (1976) has shown that excised pea apices incubated for short time periods (2 hours) with either [^{35}S]methionine or [^{14}C]-labelled amino acid mixture give rise to similar patterns of labelled polypeptides. In vivo-labelled leaf polypeptides were divided

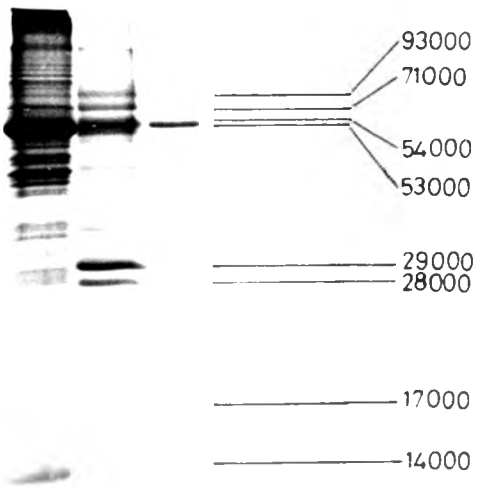
into soluble and insoluble fractions (Section II 2A) and analysed by electrophoresis on Chua SDS polyacrylamide-gradient slab gels (Section II 2F(i)b). These gels resolve polypeptides of between 14 000 and 128 000 molecular weight on a single gel as shown, for example, in Figure 14. However, it is not possible to construct a calibration curve for the estimation of molecular weights of unknown polypeptides resolved on gradient gels. Molecular weights were therefore estimated from the mobility of purified proteins of known molecular weight on a Chua 15% polyacrylamide gel. Figure 13A shows the calibration curve obtained from such a gel. The curve consists of two straight lines which meet at around 53 000 molecular weight. Similar biphasic plots have been reported by Neville (1971) and Eaglesham and Ellis (1974).

The pattern of soluble and insoluble leaf polypeptides on a Chua 15% polyacrylamide gel is shown in Figure 13B. The most heavily stained polypeptides revealed by Coomassie Blue in the soluble protein fraction (track i) have molecular weights of 53 000 and 14 000. These two polypeptides co-electrophorese with LSU and SSU respectively of Fraction I protein (track iii). The most heavily stained polypeptides in the insoluble protein fraction (track ii) have estimated molecular weights of between 93 000 and 17 000. There are also many minor stained components in both fractions. Although this gel is suitable for use in the estimation of molecular weights of polypeptides, the resolution is poor compared to gels containing a gradient of polyacrylamide concentration. Chua SDS polyacrylamide gradient gels were therefore used for the remainder of this section.

A


Relative mobility
0.5
0
4.0
4.5
5.0
Log [molecular weight]

B


93000
71000
54000
53000
29000
28000
17000
14000

(i) (ii) (iii)

Figure 13

Molecular weight calibration of spinach primary leaf polypeptides.

The mobility of nine standard proteins on a 15% Chua SDS-polyacrylamide slab gel (Section II 2F(i)b) was measured relative to the bromophenol blue front. The $\log_{10}$ of molecular weight was plotted against relative mobility to give a calibration curve from which the apparent molecular weight of spinach leaf polypeptides could be estimated. The following molecular weight values were used:-

BSA (68 000);
LSU (55 000);
ovalbumin (43 000);
carbonic anhydrase (29 000);
chymotrypsin (25 700);
trypsin (23 300);
soybean trypsin inhibitor (21 500);
myoglobin (17 200);
cytochrome c (12 500).

A. Molecular weight calibration curve
B. Equivalent loadings of
   (i) soluble spinach primary leaf protein
   (ii) insoluble spinach primary leaf protein
   (iii) spinach Fraction I protein marker

The pattern of soluble and insoluble leaf polypeptides from compost-grown and hydroponically-grown plants on a Chua gradient gel is shown in Figure 14. The separation of soluble polypeptides (tracks B and E) from insoluble polypeptides (tracks C and F) is complete with the exception of a few polypeptides. The major polypeptides present in both soluble and insoluble protein fractions have the mobilities of the α and β subunits of CF_1. The apparently incomplete fractionation of these two polypeptides may be due to their loose attachment to thylakoid membranes rather than cross-contamination of soluble and insoluble polypeptides. The complete fractionation of many other polypeptides in the same preparation is consistent with this explanation. Complete fractionation is an important prerequisite for the estimation of ratios of incorporation of isotope into soluble polypeptides relative to insoluble polypeptides. It is also evident from Figure 14 that protein fractions from compost-grown (Figure 14A) and hydroponically-grown (Figure 14B) leaves have identical stained band patterns. This finding is consistent with the results in Section III.1, showing that the growth of hydroponically-grown and compost-grown plants is similar.

An autoradiogram of a gel of soluble and insoluble protein fractions from compost-grown primary leaves is shown in Figure 15A. Many of the stained bands visible in Figure 14 (tracks B and C) are labelled. Most of the labelled bands are unidentified. The following polypeptides, which are heavily labelled, have been identified by comparison of their electrophoretic mobilities with those of known marker proteins:-

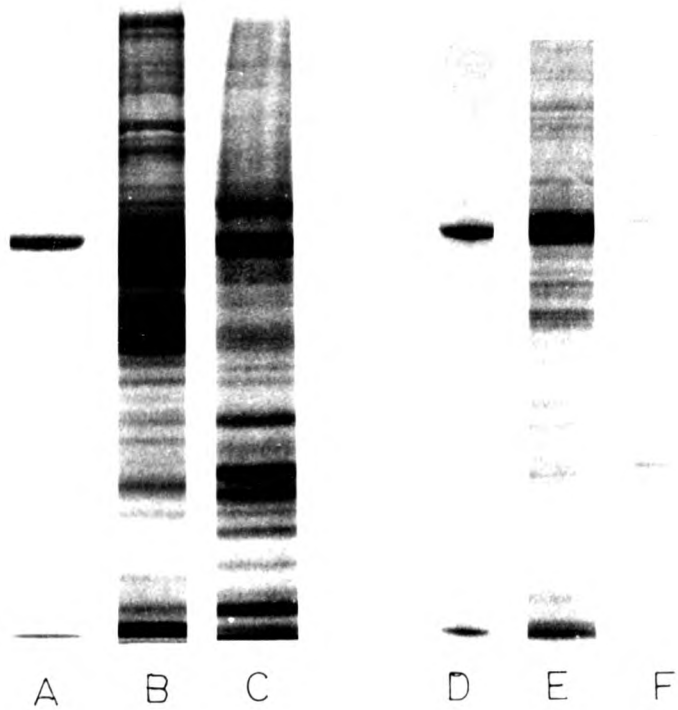

A
B
C
D
E
F

Figure 14

Electrophoretic analysis of soluble and insoluble protein fractions prepared from spinach primary leaves.

Proteins from compost-grown or hydroponically-grown spinach primary leaves were divided into soluble and insoluble protein fractions (Section II 2A). These protein fractions were analyzed by electrophoresis on a Chua SDS-polyacrylamide slab gel (Section II 2F(i)b) containing a linear polyacrylamide gradient of 7.5-15%. Polypeptide bands were visualised by staining in Coomassie Brilliant Blue (Section II 2F(i)b) and dried down by the method described in Section II 2K.

A. Purified spinach Fraction I protein marker

B. Soluble protein from compost-grown leaves

C. Insoluble protein from compost-grown leaves

D. Purified spinach Fraction I protein marker

E. Soluble protein from hydroponically-grown leaves

F. Insoluble protein from hydroponically-grown leaves

Tracks B and C, and tracks E and F were loaded with samples representing equal percentages of leaf extract.

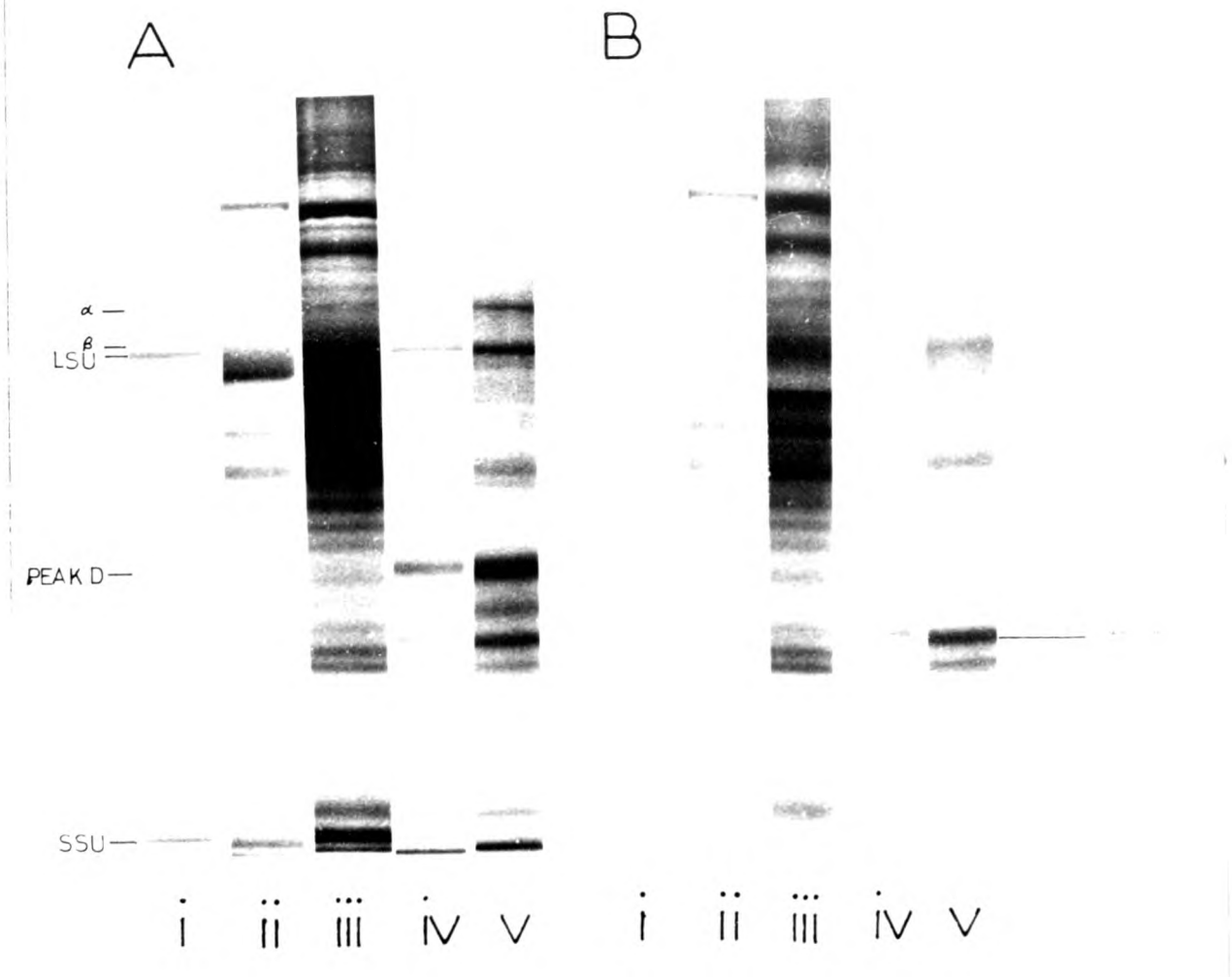

A
B
α
β
LSU
PEAK D
SSU
i
ii
iii
iv
v
i
ii
iii
iv
v

Figure 15

Comparison of [^{35}S]-labelled soluble and insoluble protein fractions prepared from spinach primary leaves radiolabelled in the presence and absence of D-threo chloramphenicol.

[^{35}S]methionine-labelled proteins compost-grown spinach primary leaves were prepared (Section II 2C) and analysed by the procedure described in Figure 14. The dried down polyacrylamide gels were autoradiographed for 7 days. Gel A shows polypeptides from leaves incubated with [^{35}S]methionine solution only; gel B shows the polypeptides from leaves incubated with [^{35}S]methionine solution and 50 µg/ml D-threo chloramphenicol.

A. (i) Purified spinach [^{14}C]-labelled Fraction I protein marker
(ii) Soluble protein
(iii) Soluble protein
(iv) Insoluble protein
(v) Insoluble protein

B. (i) Purified spinach [^{14}C]-labelled Fraction I protein marker
(ii) Soluble protein
(iii) Soluble protein
(iv) Insoluble protein
(v) Insoluble protein

Tracks A(ii), A(iv), B(ii) and B(iv) were loaded with samples representing equal percentages of leaf extract. Tracks A(iii), A(v), B(iii) and B(v) were loaded with five times the samples loaded in A(ii) and A(iv).

Chl a/b = chlorophyll a/b binding protein

CHL a/b

1. the large and small subunits of Fraction I protein;
2. peak D;
3. the α and β subunits of CF_1;
4. the light-harvesting chlorophyll a/b binding protein.

To confirm these identifications, the labelling experiments were repeated in the presence of inhibitors of chloroplast and cytoplasmic ribosomes. The large and small subunits of Fraction I protein are both heavily labelled in the soluble protein fraction in the absence of inhibitors (Fig. 15A, tracks ii and iii). The large subunit is not labelled in the presence of chloramphenicol whilst there is still incorporation into the small subunit (Fig. 15B tracks ii and iii). The pattern of labelling of these two polypeptides is consistent with findings from other studies discussed in Section I.3. Peak D is a major labelled component of the insoluble protein fraction (Fig. 15, tracks iv and v). This polypeptide is not labelled in the presence of chloramphenicol (Fig. 15B, tracks iv and v); this observation is consistent with its synthesis in isolated chloroplasts (Eaglesham and Ellis, 1974) and its encoding in the chloroplast genome (Coen et al., 1978). The molecular weight and pattern of labelling of this polypeptide with respect to inhibitors was therefore used for identification.

The insoluble protein fraction also contains two polypeptides having the mobilities of the α and β subunits of CF_1, (Fig. 15A, tracks iv and v). To check this point, spinach CF_1 was prepared by the method of Strotmann et al. (1976) which utilizes a low salt concentration to remove CF_1 from washed thylakoids. This treatment yields an enriched preparation of CF_1. Figure 16 shows a comparison of thylakoid total protein (track A) with CF_1 polypeptides washed from the

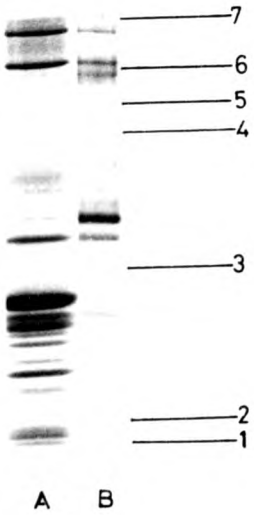

7
6
5
4
3
2
1
A
B

Figure 16

Comparison of proteins extracted from washed thylakoids with standard proteins.

Washed chloroplasts were prepared from spinach primary leaves by the method described in Section II 2D(i) and a CF_1 fraction prepared by the procedure in Section II 2M. The extract was analyzed on a Chua 7.5-15% polyacrylamide gradient gel (Section II 2F(i)b). A portion of the sodium pyrophosphate washed membranes were also analyzed in this way. Polypeptides were visualised by staining with Coomassie Blue dye.

A. Thylakoid polypeptides from sodium pyrophosphate-washed membranes containing 20 µg of chlorophyll.

B. Sucrose-Tricine-extracted CF_1 polypeptides washed from thylakoids containing 500 µg chlorophyll.

Molecular weight standards:-

1. haemoglobin (15 500);
2. myoglobin (17 200);
3. trypsin (23 000);
4. chymotrypsin (25 000);
5. ovalbumin (43 000);
6. LSU (55 000);
7. BSA (68 000).

thylakoid preparation shown in track A (track B). Track B confirms the relative mobility of these polypeptides and the resolution of the β polypeptide from LSU on the gel system used.

Hydroponically-grown leaves labelled *in vivo* with [^{35}S] methionine have a pattern of labelled soluble and insoluble polypeptide bands which is similar to that found for compost-grown plants (Figure 17, tracks B and C). Radiolabelling of leaves in chloramphenicol abolishes incorporation of isotope into LSU (track D), peak D and the α and β subunits of CF_1 (track E). In contrast, when leaves are labelled in the presence of cycloheximide, the pattern of incorporation is that shown in Figure 18. In the soluble protein fraction (tracks B and E), LSU is the most heavily labelled polypeptide; in the insoluble protein fraction (track F), peak D and the α and β CF_1 polypeptides are labelled. However, there is a marked reduction in incorporation of isotope into one of the heavily stained insoluble polypeptides. This polypeptide, which has a mobility corresponding to a molecular weight of about 28 000 is likely to be the light-harvesting chlorophyll *a/b* binding protein, a cytoplasmically-synthesized thylakoid protein (Section I.2c).

In summary, spinach primary leaves incorporate isotope into a number of chloroplast polypeptides. Some of these polypeptides have been identified by comparison with purified marker polypeptides, and by their response to treatment with inhibitors of protein synthesis. These polypeptides which are synthesized inside the chloroplast are the particular concern of the present work. It is of interest to know whether these polypeptides are synthesized continually at a constant rate throughout choroplast development, or whether their synthesis is confined to specific periods.

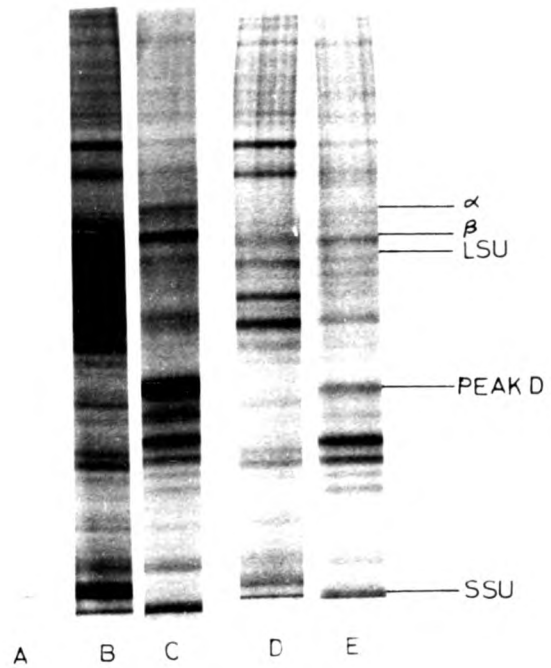

α
β
LSU
PEAK D
SSU
A
B
C
D
E

Figure 17

Electrophoretic analysis of [^{35}S]-labelled soluble and insoluble protein fractions prepared from hydroponically-grown spinach primary leaves labelled in the presence and absence of D-threo chloramphenicol.

[^{35}S]-labelled proteins from hydroponically-grown spinach primary leaves were prepared (Section II 2C) and analysed by the procedure described in Figure 15.

Tracks A - E show autoradiographs of

A. Purified [^{14}C]-labelled spinach Fraction I protein marker.

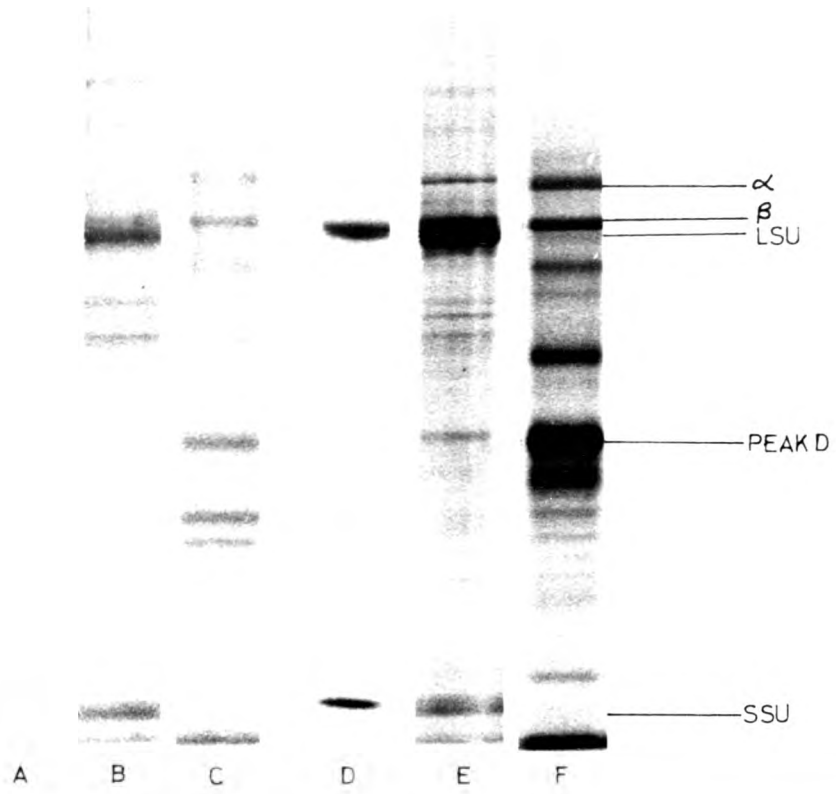
B. Soluble protein.

C. Insoluble protein.

D. Soluble protein.

E. Insoluble protein.

The leaves were incubated either in [^{35}S]methionine solution (tracks B and C) or in isotope solution containing 50 µg/ml D-threo chloramphenicol (tracks D and E). Tracks B-E were loaded with samples representing equal percentages of leaf extract.


α
β
LSU
PEAK D
SSU
A
B
C
D
E
F

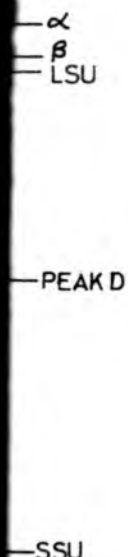


Figure 18

Comparison of *in vivo* [^{35}S]methionine-labelled proteins prepared from hydroponically-grown spinach primary leaves radiolabelled in the presence and absence of cycloheximide.

[^{35}S]-labelled protein prepared from leaves radiolabelled in [^{35}S]methionine solution (tracks B and C) or [^{35}S]methionine solution containing 4 µg/ml cycloheximide were analysed as described in Figure 15. Tracks B, C, E and F were loaded with samples containing equal percentages of leaf extract. Tracks A, B and C were autoradiographed for 63 hours; tracks D, E and F were autoradiographed for 72 hours.

A. [^{14}C]-labelled purified spinach Fraction I protein marker.

B. Soluble protein.

C. Insoluble protein.

D. [^{14}C]-labelled purified spinach Fraction I protein marker.

E. Soluble protein.

F. Insoluble protein.

B. Quantitation of the incorporation of [^{35}S]methionine into the two major products of chloroplast protein synthesis

Having established that leaves detached from both hydroponically-grown and compost-grown plants incorporate labelled methionine into the major products of chloroplast protein synthesis *in vivo*, changes in the pattern of labelling of these polypeptides during growth were measured. Figure 19 shows the pattern of polypeptides labelled *in vivo* in leaves detached from compost-grown plants 18, 25, 30 and 37 days after germination. Labelling was carried out at the same time under fixed conditions at each time point, using plants and isotope taken from a single batch. The tracks shown in Figure 19 contain equal trichloroacetic acid-insoluble radioactivity. An increased incorporation of isotope into any particular polypeptide relative to the total incorporation will therefore result in an increased blackening of the autoradiograph for that polypeptide band. Thus, the following points can be seen in Figure 19:-

1. with increasing leaf age, there is a decrease in the labelling of LSU relative to other soluble polypeptides and a concomitant decrease in incorporation into SSU (tracks B-E);
2. there is a decrease in incorporation of isotope into the chlorophyll *a*/*b* binding protein during leaf development;
3. incorporation of isotope into peak D increases markedly during leaf development so that it becomes the predominant labelled insoluble polypeptide in 37 day-old leaves;
4. the changes in incorporation of [^{35}S]methionine *in vivo* during leaf development are quantitative rather than qualitative. That is, the spectrum of polypeptides synthesized during leaf development does not change radically, but there are considerable variations in the amountof a polypeptide synthesized at any given time.

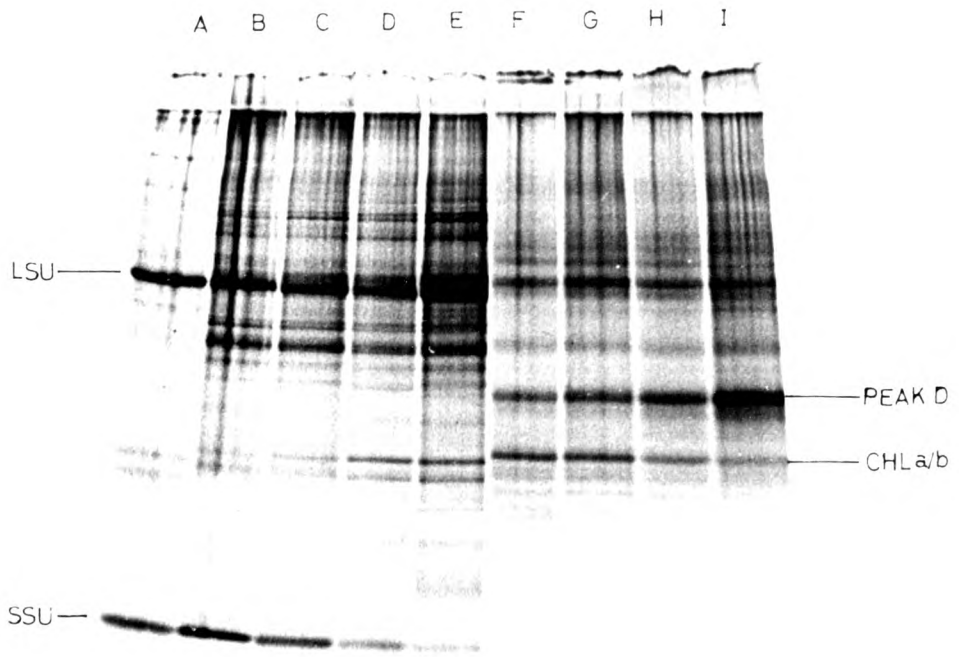

A
B
C
D
E
F
G
H
I
LSU
PEAK D
CHL a/b
SSU

Figure 19

Changes in the pattern of proteins labelled with [^{35}S] methionine during spinach primary leaf development.

Primary leaves from compost-grown spinach plants were radiolabelled *in vivo* with [^{35}S] methionine by the method described in Section II 2A and analysed on a Chua-type linear 7.5-15% polyacrylamide gradient SDS-gel. The gel was dried down and autoradiographed. Tracks were loaded with samples representing equal trichloroacetic acid-insoluble incorporation. Plants of increasing age were taken from a single batch and the same [^{35}S] methionine stock was used throughout the experiment.

Track A. Purified [^{14}C] labelled Fraction I protein.
B. Soluble proteins from 18 day leaves.
C. Soluble proteins from 25 day leaves.
D. Soluble proteins from 30 day leaves.
E. Soluble proteins from 37 day leaves.
F. Insoluble proteins from 18 day leaves.
G. Insoluble proteins from 25 day leaves.
H. Insoluble proteins from 30 day leaves.
I. Insoluble proteins from 37 day leaves.

Figure 20 shows the pattern of polypeptides labelled *in vivo* in the presence of chloramphenicol. The leaves were detached from the same batch of plants used for the data shown in Figure 23. Chloramphenicol abolishes incorporation of [^{35}S]methionine into LSU (tracks B, C, D and E) and peak D (tracks F, G, H and I). However, the change in incorporation of isotope into the chlorophyll *a*/*b* binding protein observed during leaf development (Figure 19) is unaffected by chloramphenicol (tracks F, G, H and I). This polypeptide is not a product of chloroplast protein synthesis, nor is its synthesis directly dependent on the synthesis of other chloroplast polypeptides. Similar findings have been reported for pea by Cashmore (1976) using chloramphenicol and cycloheximide, and by Ellis (1975) using MDMP and lincomycin. The data obtained thus far indicate that there are changes in the *in vivo* labelling of chloroplast proteins which are known products of chloroplast protein synthesis. These polypeptides,(LSU, peak D and the α and β subunits of CF_1), are therefore potentially useful for quantitation of the changes in chloroplast protein synthesis during development.

Quantitation of the changes in synthesis of these polypeptides by absolute measurement of the amount of isotope incorporated at each time point is subject to a number of possible criticisms. It is likely that different intracellular compartments contain different concentrations and amounts of methionine; these parameters could change with time during leaf development. A further source of difficulty may be changes in the rate of methionine transport across both the plasma membrane and the chloroplast envelope. These problems can be avoided by measuring the relative incorporation of isotope into two

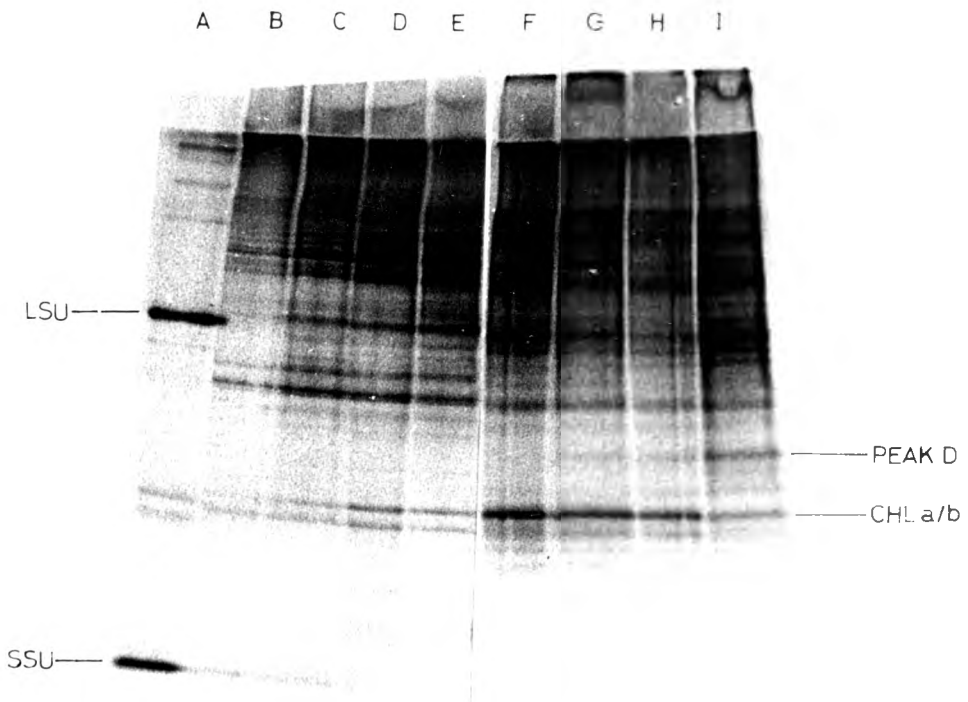

A
B
C
D
E
F
G
H
I
LSU
PEAK D
CHL a/b
SSU

Figure 20

Changes in the pattern of proteins labelled with [^{35}S] methionine in the presence of D-threo chloramphenicol during spinach primary leaf development.

Primary leaves were radiolabelled and the soluble and insoluble protein fractions analysed by the procedure set out in Figure 19, except that 50 µg/ml D-threo chloramphenicol was included in the isotope feeding solution. Tracks were loaded with samples representing equal TCA-insoluble incorporation, the gel dried down and autoradiographed.

Track A. Purified [^{14}C]-labelled Fraction I protein.
B. Soluble proteins from 18 day leaves.
C. Soluble proteins from 25 day leaves.
D. Soluble proteins from 30 day leaves.
E. Soluble proteins from 37 day leaves.
F. Insoluble proteins from 18 day leaves.
G. Insoluble proteins from 25 day leaves.
H. Insoluble proteins from 30 day leaves.
I. Insoluble proteins from 37 day leaves.

chloroplast proteins, with the following provisos:-

1. the polypeptides must contain sufficient methionine to give a band on an autoradiograph;

2. both polypeptides must be products of protein synthesis inside the chloroplast. A comparison between a cytoplasmically-synthesized polypeptide and a chloroplast-synthesized polypeptide is not meaningful because the methionine pools inside and outside the chloroplast are likely to differ. However, the assumption that there is a single methionine pool within the chloroplast is not unreasonable since both soluble ribosomes and bound ribosomes are in contact with the stromal compartment;

3. the polypeptides chosen for quantitation must be either soluble or insoluble within the working definition discussed in Section III.1, otherwise estimation of incorporation from gels of these fractions becomes difficult. This proviso excludes the α and β subunits of CF_1 as possible $^{poly}_{A}$peptides for quantitation since, under the extraction conditions employed, these two polypeptides are both soluble and insoluble. For these reasons, LSU and peak D were chosen for quantitation. Figure 21 shows densitometer traces of autoradiographs from gels of *in vivo*-labelled spinach polypeptides. For estimation of the relative incorporation of isotope into LSU and peak D, peak height rather than peak area was used because it is difficult to define the width of peak bases. The scans obtained are quantitative for both soluble and insoluble proteins. For example, a doubling of the loading of soluble protein results in an increase in LSU peak height from 14 mm to 28 mm (*cf.* track $A_{(i)}$ with track $A_{(ii)}$ in Fig. 21). By scanning a range of loadings (normally 4) of each sample, it is possible to construct a table of ratios of incorporation of isotope into LSU relative to peak D during leaf development. These data are listed

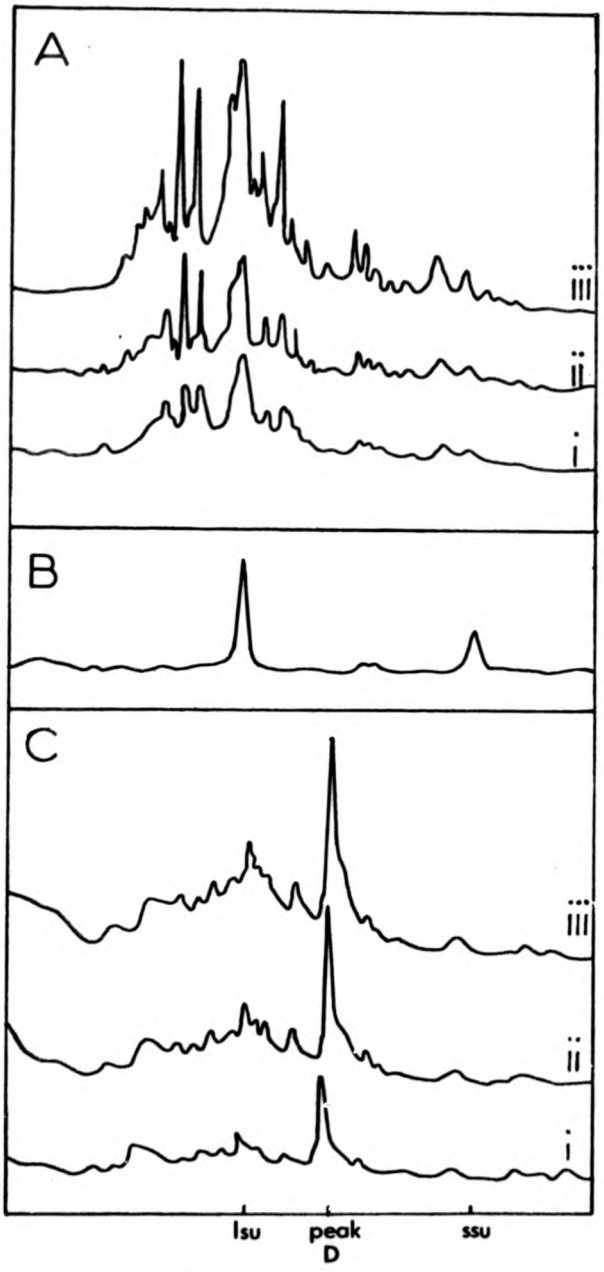

A
iii
ii
i
B
C
iii
ii
i
lsu
peak
D
ssu

Figure 21

Quantitation of the relative incorporation of [^{35}S]methionine into LSU and peak D using densitometer traces.

Compost-grown spinach primary leaves from 48 day old plants were *in vivo*-labelled with [^{35}S]methionine (Section II 2C) and soluble and insoluble protein fractions prepared by the method described in Section II 2A. These protein fractions were analysed by electrophoresis on 7.5-15% Chua linear gradient polyacrylamide gels (Section II 2F(i)b), dried down and autoradiographed as described in Section II 2K. The autoradiograph was exposed for 7 days and it was then scanned on a Joyce-Loebl densitometer.

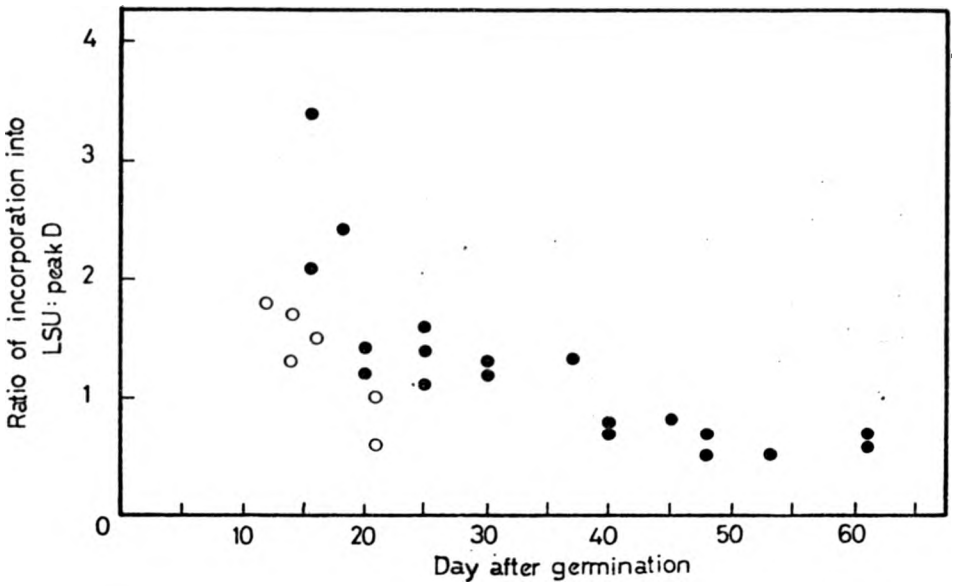
A. Total soluble protein.

B. Purified [^{14}C]-labelled spinach Fraction I protein.

C. Total insoluble protein.

Tracks A(i) and C(i) are loaded with samples representing equal percentages of leaf extract. Track (ii) was loaded with twice the sample loaded on track (i); track (iii) was loaded with three times the sample on track (i).

A.


Ratio of incorporation into LSU: peak D
0
1
2
3
4
10
20
30
40
50
60
Day after germination

B.

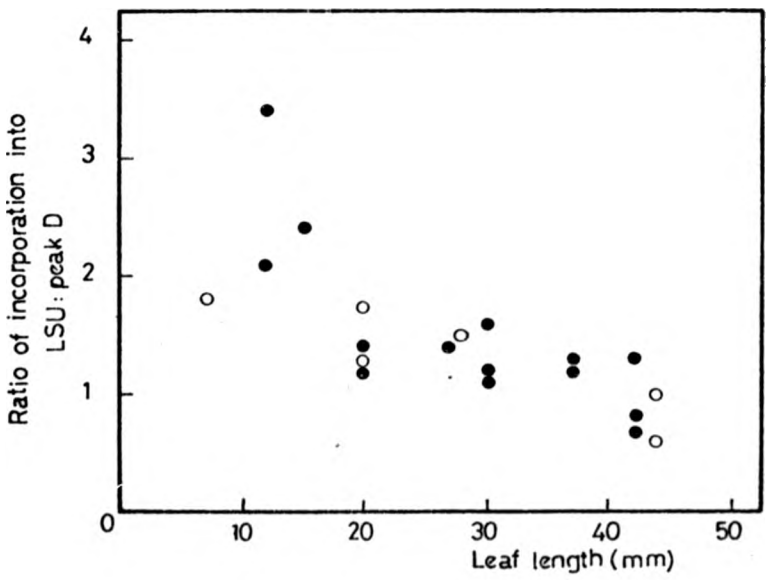

Ratio of incorporation into LSU: peak D
0
1
2
3
4
10
20
30
40
50
Leaf length (mm)

Figure 22

Ratios of incorporation of [^{35}S]methionine into LSU relative to peak D during spinach primary leaf development.

Compost-grown and hydroponically-grown spinach leaf proteins were analysed as in Figure 14. The values shown for each ratio were measured from different gel loadings at each time point. The data are taken from four experiments.

O——O hydroponically-grown leaves

●——● compost-grown leaves

A. Ratio of LSU:peak D against days after germination

B. Ratio of LSU:peak D against leaf length

in Table I. The ratio of incorporation of [^{35}S] methionine into LSU relative to peak D *in vivo* decreases 4-fold over a period of about 43 days. These figures confirm the visual impression of the autoradiographic pattern shown in Figure 19. A similar decrease in this ratio is also found in hydroponically-grown leaves during leaf development. However, the change in ratio measured in hydroponically-grown plants is greater than in compost-grown plants over a similar time period. Figure 22 shows a comparison of the ratios of incorporation of isotope into LSU relative to peak D in compost-grown and hydroponically-grown leaves during leaf development. It can be seen that although the rate of change of ratio differs on a time basis (Figure 22a), the change is similar on a leaf length basis (Figure 22b). Thus, as was concluded in Section III.1, the pattern of growth for the two types of plant is similar.

In conclusion, it is possible to label products of chloroplast protein synthesis *in vivo* with [^{35}S]methionine. Analysis of soluble and insoluble leaf polypeptides on polyacrylamide gels indicates that there is little change in the spectrum of polypeptides synthesized. Rather, there are quantitative changes in incorporation of isotope into these polypeptides.

C. Discussion

Incubation of excised spinach primary leaves with [^{35}S] methionine results in the incorporation of isotope into many polypeptides, the majority of which are unidentified. However, there are two major points which emerge from the pattern of labelling of known chloroplast polypeptides:-

1. the qualitative pattern of incorporation of [^{35}S] methionine into leaf polypeptides does not change during the period of development studied; that is, the number of labelled bands does not change;

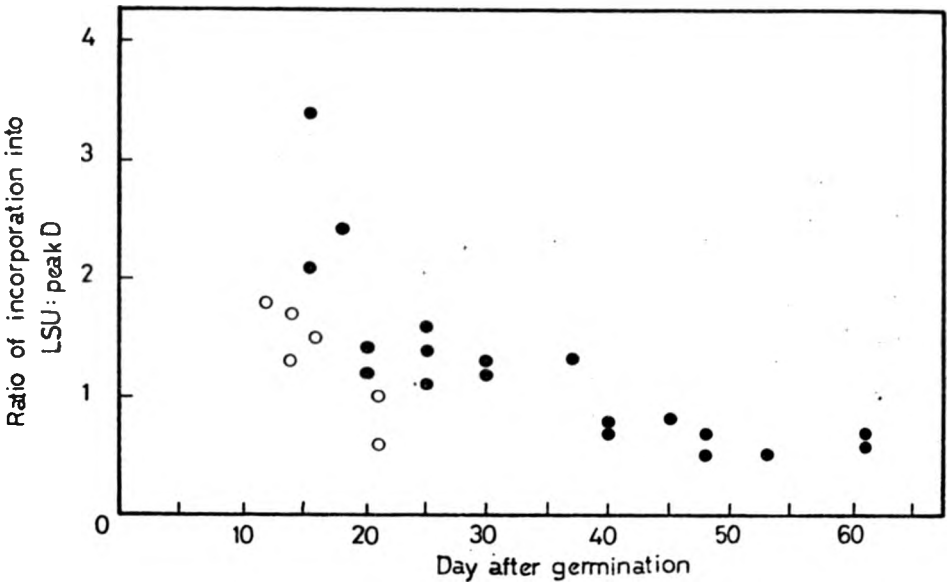
	Day after germination	Leaf length	Ratio of incorporation into LSU:peak D
Compost-grown plants	16	12	2.1, 3.4, 4.3
	16	15	2.4
	19	20	1.4, 1.2
	25	27	1.4
	25	30	1.1, 1.2, 1.6
	30	37	1.3
	30	37	1.2, 1.3
	37	42	1.3
	40	42	0.7, 0.7, 0.8
	45	-	0.8
	48	-	0.5, 0.7
	53	-	0.5, 0.5, 0.5
	61	-	0.7, 0.6
Hydroponically-grown plants	12	7	1.8, 1.8
	14	20	1.4, 1.8
	16	28	1.5, 1.5
	21	44	0.6, 1.0

Table I

Calculated ratios of [^{35}S] methionine incorporation into LSU relative to peak D during the development of spinach primary leaves.

Soluble and insoluble [^{35}S] methionine-labelled leaf polypeptides were prepared by the method described in Section II 2A and analysed on Chua-type 7.5-15% polyacrylamide gels (Section II 2F(i)b). Autoradiographs were scanned as described in Figure 21 and values for the ratio LSU:peak D calculated from different loadings. Each value is for a single loading. Data are from four experiments, each experiment using a separate batch of plants.

A.


Ratio of incorporation into LSU: peak D
0
1
2
3
4
10
20
30
40
50
60
Day after germination

B.

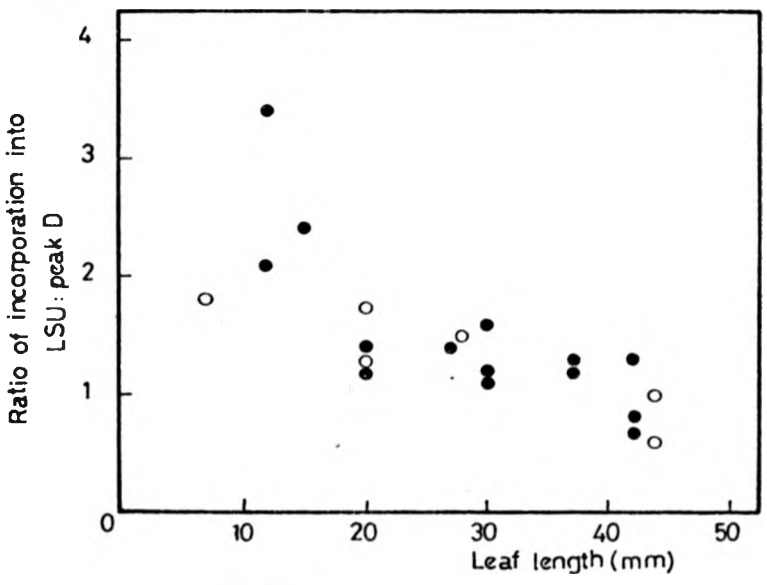

Ratio of incorporation into LSU: peak D
0
1
2
3
4
10
20
30
40
50
Leaf length (mm)

Figure 22

Ratios of incorporation of [^{35}S]methionine into LSU relative to peak D during spinach primary leaf development.

Compost-grown and hydroponically-grown spinach leaf proteins were analysed as in Figure 14. The values shown for each ratio were measured from different gel loadings at each time point. The data are taken from four experiments.

O——O hydroponically-grown leaves

●——● compost-grown leaves

A. Ratio of LSU:peak D against days after germination

B. Ratio of LSU:peak D against leaf length

2. there is a quantitative change in incorporation of isotope into LSU and peak D.

These points will now be discussed.

The major problem in this section is the identification of *in vivo*-labelled polypeptides. There are several chloroplast proteins which have been identified from their enzymic activities, for example triosephosphate dehydrogenase (Heber *et al.*, 1963) or from physical properties, for example cytochrome b_{559} (Zielinski and Price, 1979). Many of these proteins are relatively minor components and have not been located on gels. This study has therefore concentrated on known proteins which can be visualised by gel electrophoresis of crude extracts. The criteria used in this study for matching an *in vivo*-labelled polypeptide to a previously characterized polypeptide are as follows:-

1. mobility on SDS-polyacrylamide gels relative to polypeptides of known molecular weight;
2. co-electrophoresis with purified standard proteins;
3. solubility properties;
4. labelling in the presence of D-*threo* chloramphenicol or cycloheximide.

The use of mobility on gels as the sole criterion for identification is insufficient since this is a property which can be shared by several unrelated polypeptides. However, division of leaf polypeptides into soluble and insoluble fractions and the use of inhibitors are additional criteria which, taken together, provide reasonable evidence for identification. A fuller discussion of the problems of polypeptide identification is made in Section IVB.

In vivo-labelling studies of green plants have been made in bean (Machold and Aurich, 1972) and pea (Cashmore, 1976). The pattern of Coomassie Blue-stained bands in soluble and insoluble pea leaf polypeptide fractions is similar, although not identical to those obtained in spinach in this study. In short-term (2 hour) in vivo-labelling experiments with [^{35}S] methionine, four major polypeptide bands are labelled in pea. The large and small subunits of Fraction I protein are the major labelled soluble polypeptides, whilst the two major labelled insoluble polypeptides are thylakoid polypeptides, PI and PII. Gillham et al. (1978) postulate that Cashmore's PI, which has a molecular weight of 58 000, is a poorly-resolved doublet of the α and β subunits of CF_1. The polypeptide PII has a mobility similar to that of the pea chlorophyll a/b binding protein. It is interesting that the synthesis of PI in pea is affected when in vivo labelling is carried out in either D-threo chloramphenicol or cycloheximide (Cashmore, 1976). Inclusion of D-threo chloramphenicol results in the inhibition of synthesis of the PI band whilst cycloheximide treatment causes about 50% of labelled PI to appear in the soluble fraction. Gillham et al. suggest that if PI does represent α and β CF_1 subunits, then the lack of assembly of α and β subunits into CF_1 may reflect a deficiency of γ and δ CF_1 subunits. The synthesis of both the γ and δ polypeptides is cycloheximide-sensitive (Bouthyette and Jaggendorf, 1978). Figure 22 of the present study is consistent with Cashmore's data, although the data are difficult to quantitate, mainly because CF_1 is partially soluble in the grinding buffer.

Machold and Aurich (1972) have carried out both long term (25 hours) and short term (5 hours) labelling experiments with

partially-greened, excised Vicia faba shoots. Shoots incubated with [^{14}C]-protein hydrolysate and [^{14}C]-leucine incorporated label into 21 lamellar polypeptides, and 12 of these were not synthesized in the presence of D-threo chloramphenicol. The synthesis of CPI, (their band B), was found to be strongly inhibited by chloramphenicol but also to some extent by cycloheximide. They interpret these data to imply that a major CPI component is synthesized in the chloroplast, but that a subunit may be synthesized in the cytoplasm. Ellis (1975) finds a similar pattern of labelling of CPI in pea apices, using MDMP and lincomycin. However, he interprets the labelling pattern as indicating the requirement of a chloroplast-synthesized polypeptide to insert cytoplasmically-synthesized CPI into chloroplast membranes. The synthesis of the CPII polypeptide, the chlorophyll a/b binding protein has been found to be inhibited by cycloheximide in pea (Cashmore, 1970), MDMP in pea (Ellis, 1975) and by cycloheximide in Vicia faba (Machold and Aurich, 1972). Since this polypeptide is not synthesized in isolated chloroplasts (Section I 2C), it is likely to be a product of cytoplasmic protein synthesis. The data in this thesis are consistent with this conclusion in that the synthesis of the chlorophyll a/b binding protein is inhibited by cycloheximide (Fig. 18) but not by chloramphenicol (Fig. 17). Thus, the use of inhibitors has been informative in the case of CPII synthesis but is insufficient to resolve the sites of synthesis of CPI polypeptides. The use of another approach, such as the in vitro reconstitution of synthesis of these complexes, is needed to resolve this point.

Grebanier et al. (1978) have shown that, in maize, a polypeptide of M. wt. 32 000 is synthesized as a precursor of 34 000,

although these two molecules are poorly resolved on gels of *in vivo* labelled polypeptides. A 32 000 M. wt. thylakoid polypeptide in *Spirodela* is also synthesized as a 33 500 M. wt. precursor which is rapidly processed *in vivo* (Edelman and Reisfeld, 1978). In the present study, there is no evidence that peak D is made as a precursor *in vivo*. This could be for the following reasons:-

1. the gel system used does not resolve the two polypeptides because of their similar molecular weights and heavy incorporation;
2. processing is rapid enough to convert all the precursor into 32 000 M. wt. polypeptides.

Synthesis of peak D in a heterologous cell-free extract programmed with chloroplast mRNA is needed to resolve this point. If peak D is initially made as a larger precursor in spinach primary leaves, then the cell-free extract should synthesize this polypeptide, but be unable to process it.

The major conclusion from this section is that the changes in protein synthesis taking place during spinach primary leaf development are quantitative rather than qualitative. However, although no major labelled bands appear or disappear during the period of development studied, this does not necessarily imply that the spectrum of polypeptides synthesized is unchanging. There may be qualitative changes in protein synthesis which are beyond the resolution of the gel system used. For example, there may be regulatory polypeptides which function to co-ordinate chloroplast and cytoplasmic protein synthesis. If these polypeptides were synthesized in small amounts during chloroplast development, a two-dimensional gel system would be necessary to detect them. The other point to consider is the time scale of

leaf development. The plants used in this study were in the period of development which spans the period of primary leaf expansion and maturation. Major changes in the spectrum of polypeptides synthesized may only occur in very young or in senescent leaves. It could be argued, therefore, that in the period of leaf development being studied, chloroplast replication from existing mature plastids by fission and chloroplast maturation are the major events (Section I.3). Such events involve the synthesis and maintenance of existing chloroplast components rather than a shift to the synthesis of new polypeptides. On this argument, it is not surprising if there is little variation in the products of protein synthesis during leaf development.

The developmental transition of etioplast to chloroplast has often been used as a model for chloroplast development from proplastids (Section I.3). From work with greening peas, it was initially concluded that the changes in protein synthesis during etiochloroplast development are largely quantitative (Siddell and Ellis, 1975). However, use of a more sensitive gel system has revealed that there are both quantitative and qualitative changes in the proteins synthesized in developing maize etioplasts (Grebanier et al , 1979). In maize etioplast development, two major polypeptides of etioplasts disappear during greening, whilst bands corresponding to the mobilities of the chlorophyll a/b binding protein, and 32 000 polypeptide appear. The relationship of such changes to chloroplast development from proplastids is not clear. These changes may reflect the loss of etioplast-specific polypeptides and the gain of polypeptides whose synthesis requires light. It is not known

whether these changes occur during the normal development of chloroplasts from proplastids. The resolution of this problem requires biochemical studies of protein synthesis in proplastids and very early stages of chloroplast development.

Quantitative changes in protein synthesis in greening tissue has been described in three systems. An increased synthesis of peak D has been found *in vivo* in maize (Coen *et al*, 1978) and *Spirodela* (Reisfeld *et al*, 1978). In both these studies, the synthesis of peak D is dependent on light, whereas the synthesis of LSU is independent of light. In *Spirodela*, *in vivo*-labelling studies of greening fronds indicate that the onset of maximal synthesis of peak D occurs earlier than for LSU. These studies were not continued long enough to determine whether LSU synthesis continues maximially in the light or whether synthesis declines. Plastids isolated from greening peas synthesize LSU as the major product early on in development; as greening proceeds,peak D becomes the major product of protein synthesis (Siddell and Ellis, 1975). The question as to how far studies on the greening of etiolated plants contributes to our understanding of normal chloroplast development has been discussed in Section I.3. The present study confirms that:-

1. the increased synthesis of peak D relative to LSU is not an artefact of greening, but a feature of protein synthesis of chloroplasts developing in plants grown under a more normal light regime;
2. the increased synthesis of peak D is not only a feature of protein synthesis by plastids isolated from greening leaves, but also a feature of chloroplast protein synthesis *in vivo*.

In none of these studies has the function of the changes in peak D and LSU synthesis been established. Nor has it been shown

whether the changes are related to each other. However, both these polypeptides are known to be encoded in the chloroplast genome. It is therefore of interest to ask how changes in the expression of these two genes are brought about. Work described in the following two sections is aimed at studying these changes both in isolated chloroplasts, and in cell free systems programmed with chloroplast RNA.

III.3 IN VITRO LABELLING OF SPINACH CHLOROPLASTS ISOLATED FROM DEVELOPING PRIMARY LEAVES

In Section III.2, it was established that the pattern of polypeptides labelled *in vivo* in spinach primary leaves incubated with [^{35}S] methionine does not change dramatically during leaf elongation. However, there are changes in incorporation of isotope into LSU and peak D, two known products of chloroplast protein synthesis. A similar situation has previously been described by Siddell and Ellis (1975) during etio-chloroplast development in pea. In the case of chloroplast development, it could be argued that the change in ratio of incorporation of isotope into LSU relative to peak D reflects some cytoplasmic control of chloroplast protein synthesis. For example, the synthesis of LSU may be positively regulated directly or indirectly by SSU (Highfield and Ellis, 1978), a known product of 80S cytoplasmic protein synthesis (Gray and Kekwick, 1974). If this were the case, the decreasing incorporation of isotope into LSU would be a reflection of the decreasing availability of SSU and may therefore not occur in isolated chloroplasts. Similar cytoplasmic controls may also be exerted on the developing chloroplast with respect to other polypeptides synthesized on chloroplast ribosomes. To examine these possible mechanisms of control of chloroplast development, a study of proteins synthesized in isolated developing chloroplasts was initiated. The aims of this section are therefore:-

1. to analyze the products of protein synthesis by isolated spinach chloroplasts incubated with [^{35}S]methionine;
2. to quantitate the ratio of incorporation by isolated chloroplasts of isotope into LSU relative to peak D, thus enabling a comparison of *in vivo* and *in vitro* ratios.

A. Characteristics of protein synthesis by isolated spinach chloroplasts

The characteristics of protein synthesis in plastids isolated from hydroponically-grown primary leaves are discussed in this section. Efforts to obtain active plastids from compost-grown leaves were unsuccessful even when young, rapidly expanding leaves were used. Since Hartley and Ellis (1973) found that hydroponically-grown spinach showed rapid accumulation of both cytoplasmic and chloroplast ribosomal RNA's during development of the primary leaf pair, presumably a reflection of protein-synthetic requirements, hydroponically-grown tissue was used for preparation of isolated chloroplasts. Chloroplasts isolated from such leaves were resuspended in the sorbitol/Tricine medium of Bottomley et al (1974) where light-driven or ATP-driven protein synthesis in intact plastids was measured. This buffer has been used successfully in other studies of protein synthesis in isolated spinach chloroplasts prepared by differential centrifugation (Bottomley et al 1974) and isopycnic centrifugation on silica sol gradients (Morgenthaler and Mendiola-Morgenthaler, 1976). In this study, differential centrifugation was used to prepare chloroplast pellets on the basis that it is rapid, and more likely to yield active plastids than a preparation which has been through a lengthy purification procedure.

Figure 23 shows the time-course of incorporation of [^{35}S]methionine into intact spinach primary leaf plastids. Plastid preparations were routinely examined by phase microscopy, and found to be largely free of starch and whole cells, and to be between 50% and 60% intact as judged by phase-refractility (Siddell and Ellis, 1975). Such plastids showed a linear

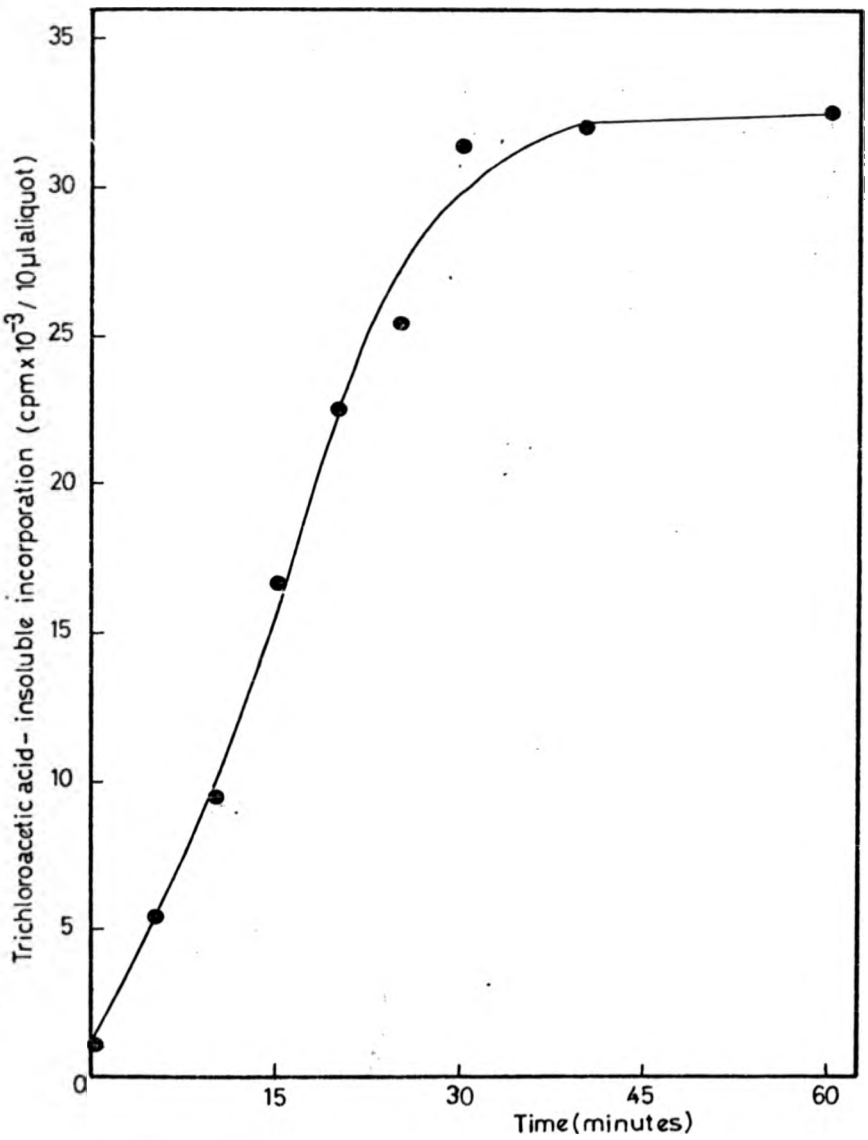

Trichloroacetic acid - insoluble incorporation (cpm x 10^{-3} / 10µl aliquot)
35
30
25
20
15
10
5
0
15
30
45
60
Time (minutes)

Figure 23

Time course of incorporation of [^{35}S] methionine by isolated spinach primary leaf chloroplasts.

Isolated chloroplasts were prepared from spinach primary leaves by the method described in Section II 2D(ii), and resuspended in sorbitol/Tricine buffer. Each 500µl incubation contained 50 µCi [^{35}S] methionine and 260 µg chlorophyll. Duplicate aliquots (10 µl) were removed at the indicated times for determination of trichloroacetic acid-insoluble incorporation (Section II 2C).

Treatment	Trichloroacetic acid-insoluble incorporation as % of control
Light (control)	100
Dark	13.6
Dark + 2 mM ATP + 0.2 mM GTP	21.5
Light + 50 µg/ml D-*threo* chloramphenicol	14.7
Light + 100 µg/ml cycloheximide	101.6
Light + 10 µg/ml RNAase	83.5

Table II

Characteristics of incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material by isolated spinach primary leaf chloroplasts.

Isolated chloroplast incubation mixtures as described in Figure 23 were incubated for 45 minutes (Section II 2D(ii)), and trichloroacetic acid-insoluble incorporation determined by the method described in Section II 2C. Incorporation into treated incubations are expressed as a percentage of the control value.

incorporation of isotope into trichloroacetic acid-insoluble material for 20 to 25 minutes, and then rapidly ceased further incorporation. This short time-course of incorporation has been found in other studies. Bottomley et al (1974) obtained linear incorporation of isotope into crude isolated spinach plastid preparations for up to 20 minutes in the light. By comparison, spinach plastids purified on silica sol gradients incorporate isotope in a linear fashion for only 10 minutes, presumably a consequence of the extra time involved in preparation (Morgenthaler and Mendiola-Morgenthaler, 1976).

The effect on protein synthesis of changing the incubation conditions is shown in Table II. There is a 7 to 8-fold stimulation in incorporation by isolated chloroplasts incubated in the light compared to that in the dark. This level of incorporation in the dark is high compared to the 3% of the light control value found by Blair and Ellis (1973) in pea, but similar values have been found in another study of spinach (Camm and Green, 1977). When ATP and GTP were included in the dark incubation, incorporation of isotope increased to 20% of the light control, a low value when compared to reported values for spinach of 61% (Bottomley et al, 1974) and for pea of 50% (Blair and Ellis, 1973). The protein synthesis inhibitor D-threo chloramphenicol (50 µg/ml) reduced incorporation of isotope to about 15% of the control value, confirming that the bulk of protein synthesis is being carried out on 70S ribosomes. In pea, the same concentration of this inhibitor was relatively more effective, giving 95% inhibition (Blair and Ellis, 1973). Bottomley et al (1974), using 100 µg/ml D-threo chloramphenicol reported only 64% inhibition. The high incorporation by isolated spinach chloroplasts in the presence of D-threo chloramphenicol

found by Bottomley et al does not appear to be the result of contamination of the plastid preparations by whole cells since cycloheximide (33 µg/ml) had no effect on incorporation in their study. Cycloheximide (100 µg/ml) had no effect on incorporation of isotope into trichloroacetic acid-insoluble material in pea (Blair and Ellis, 1973) or in this study.

Ribonuclease was used as a measure of intactness of the plastid preparation since it does not penetrate intact chloroplasts (Margulies et al , 1968; Hartley and Ellis, 1973). In this study, 84% of the incorporated label was resistant to 10 µg/ml ribonuclease (Table II), indicating that about 20% of the control incorporation is not due to intact plastids.

The overall conclusion from Table II is that chloroplasts isolated from hydroponically-grown spinach leaves are able to incorporate [^{35}S] methionine into trichloroacetic acid-insoluble material on 70S ribosomes, using light as an energy source. Since only intact plastids can use light as an energy source (Tagawa et al , 1963), and over 80% of incorporation is ribonuclease-resistant, the majority of the measured protein synthesis is taking place in intact plastids.

The pattern of polypeptides synthesized by the isolated chloroplast incubation mixtures as revealed by SDS-polyacrylamide gel electrophoresis is shown in Figure 24. Spinach chloroplasts incubated in the light with [^{35}S] methionine (tracks B and C) incorporate isotope predominantly into two polypeptides which have the mobilities of LSU and peak D. However, plastids incubated in the dark with no added energy source (tracks D and E) also incorporate isotope into polypeptides. These polypeptides are unlikely to be due to protein synthesis by microbial contaminants, since the pattern is very similar to that shown in track B,

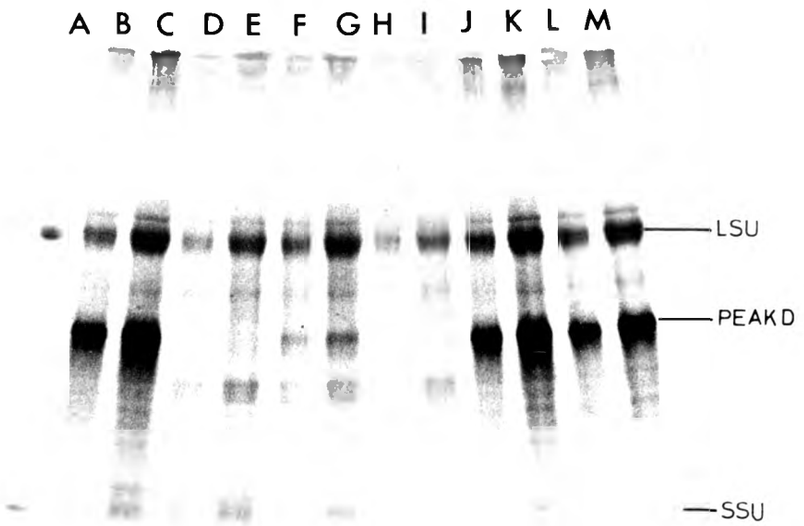


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Figure 24

The effect of various treatments on protein synthesis by isolated chloroplasts.

Spinach primary leaf chloroplasts were isolated as described in Section II 2D(i). Aliquots (0.5 ml) of chloroplast suspension in sorbitol/Tricine buffer (208 µg chlorophyll) were incubated with [^{35}S]methionine (50 µCi) as follows:-

A. [^{14}C]-labelled spinach Fraction I protein marker

B and C. Light

D and E. Dark

F and G. Dark + 2 mM ATP + 0.2 mM GTP

H and I. Light + 50 µg/ml D-threo chloramphenicol

J and K. Light + 100 µg/ml cycloheximide

L and M. Light + 10 µg/ml ribonuclease A

Tracks B, D, F, H, J and L were loaded with sample equivalent to 6 µg chlorophyll. Tracks C, E, G, I, K and M were loaded with samples equivalent to 12 µg chlorophyll. Samples were analysed on a 10-30% linear polyacrylamide gradient Chua gel containing SDS (Section II 2F(i)b). The gel was dried down and autoradiographed by the method described in Section II 2K. The gel was autoradiographed for 7 days.

although there are some differences in the relative incorporation into some polypeptides. For example, there is little, if any, incorporation into a polypeptide co-electrophoresing with peak D. It has recently been reported that darkened intact spinach chloroplasts contain significant levels of ATP in the stroma, corresponding to between 30% and 60% of the maximum ATP levels found in saturating light (Inoue et al , 1978). Thus, the low level of incorporation and concomitant synthesis of some polypeptides observed in dark-incubated intact plastids may be due, in part, to ATP present in the isolated chloroplasts. However, when exogenous ATP and GTP is supplied to plastids in the dark (Fig. 24, tracks F and G), the spectrum of polypeptides labelled is similar to that found in the light control (Fig. 24, tracks B and C), but labelled to a lesser degree. Whether this is due to impermeability of the chloroplast envelope to ATP and GTP or the relatively poor protein-synthetic activity is not clear. Bottomley et al (1974) obtained higher levels of incorporation in intact dark-incubated plastids supplied with ATP and GTP relative to the light control than was found in this study, but the polypeptide pattern was not shown. Their figures may reflect the low incorporation obtained, and the low specific activity of the isotopes used.

The effect of chloramphenicol on the labelled polypeptide pattern from isolated spinach chloroplasts analyzed on an SDS-polyacrylamide gel is shown in Figure 24, tracks H and I. The faint pattern of polypeptides labelled is a similar but reduced-intensity form of the dark pattern (tracks D and E). Cycloheximide (tracks J and K) and ribonuclease (tracks L and M) had no significant effect on the pattern of polypeptides labelled when compared to the light-control (tracks B and C), confirming

that the majority of polypeptides labelled are being synthesized on 70S ribosomes inside intact chloroplasts, which are using light as the major energy source.

The effect of lysis on trichloroacetic acid-insoluble incorporation by spinach chloroplasts is shown in Table III. In these incubations, washed, intact plastids are prepared in sucrose isolation medium (Section II 2D(ii)) and then resuspended either in sorbitol/Tricine medium (intact chloroplasts) or in low ionic strength Tricine/magnesium sulphate/2-mercaptoethanol buffer. This latter buffer lyses the plastids, resulting in complete loss of phase-brightness under the light microscope. The broken chloroplast preparation is then fractionated into soluble and membrane components, and the membrane pellet resuspended in the lysis buffer. The pH of this buffer is fixed at 8.0 because it has been estimated that the pH of the stroma of illuminated, intact spinach chloroplasts is about 8.0 (Heldt *et al*. 1973). The data in Table III show that light stimulates incorporation by intact plastids by 6 to 7-fold, confirming the results shown in Table II. When exogenous ATP and GTP are supplied to intact chloroplasts in the dark, incorporation is increased to over half the light control value. This value is higher than was observed in Table II; such values are variable, possibly because of the relatively low absolute incorporation. When the chloroplast preparation was lysed before incubation, there was a significant decrease in incorporation in the light compared to the control. This loss of incorporation by lysed chloroplast incubation mixtures in the light confirms that broken chloroplasts are unable to utilize light as an energy source (Tagawa *et al*, 1973). Both lysed and intact chloroplasts incubated in the dark incorporated

Chloroplasts	Energy source	Trichloroacetic acid-insoluble incorporation as % of control
Intact	light (control)	100
Intact	none	15.5
Intact	2 mM ATP + 0.2 mM GTP	55.2
Lysed	light	12.8
Lysed	dark	16.2
Stroma	2 mM ATP + 0.2 mM GTP	29.5
Membranes	2 mM ATP + 0.2 mM GTP	68.9

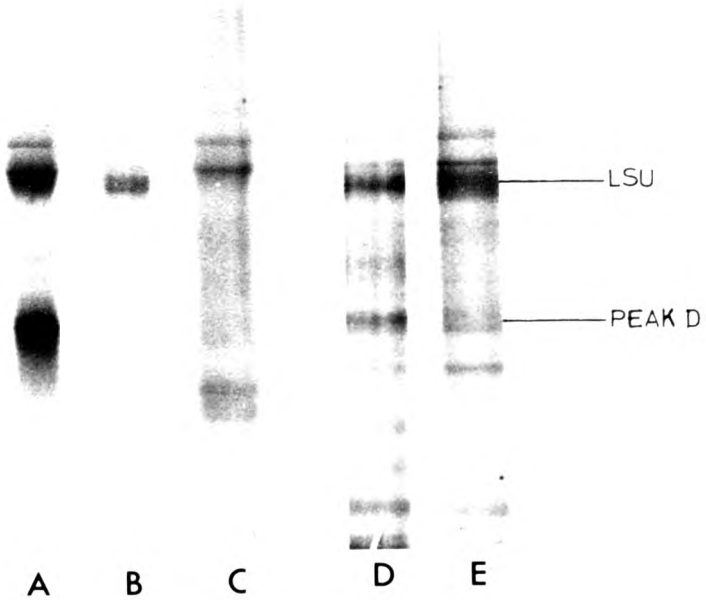
Table III

Protein synthesis by lysed and intact spinach chloroplasts.

Intact chloroplasts were prepared from spinach primary leaves by the method described in Section II 2D(i) and resuspended in either sorbitol/Tricine buffer (intact plastids) or Tricine/magnesium sulphate/2-mercaptoethanol buffer (lysed plastids) to the same final chlorophyll concentration. Incubations (1 ml) containing 88 µg chlorophyll and 100 Ci [^{35}S] methionine were incubated as described in Section II 2D(ii). Lysed plastid incubations were supplemented with 75 mM KCl (final concentration). Trichloroacetic acid-insoluble incorporation was assayed as described in Section II 2C, and values converted to percentages of the incorporation by intact chloroplasts in the light.

isotope into hot trichloroacetic acid-insoluble material, but only to a limited extent. This limited incorporation may reflect the lack of an energy supply since addition of ATP and GTP to lysed chloroplast incubation mixtures incubated in the dark resulted in increased incorporation. The membrane fraction was consistently found to incorporate more isotope than the soluble fraction. In pea, the reverse situation is observed, where a comparable fractionation yields a soluble fraction which shows almost twice the incorporation of the membrane fraction (Ellis, 1977). The relatively better incorporation of the spinach chloroplast membrane fraction compared to the stromal fraction is unexpected since the stroma should be enriched for soluble components required for protein synthesis. In pea, the protein-synthetic activity of the membrane fraction is lost on washing, presumably because washing removes fortuitously trapped soluble components (Ellis, 1977). What is clear from this study, and from similar studies of pea (Ellis, 1977) and spinach (Bottomley et al , 1974) is that the lysed chloroplasts are synthetically active to levels comparable with intact controls, when provided with ATP and GTP. This activity is surprising in view of the dilution of soluble components involved in protein synthesis which occurs on the lysis of chloroplasts. Although dilution of enzymes will not affect their rates of activity in a fixed volume, dilution of substrates would be expected to lower the reaction rate, and it is difficult to see why this does not occur (Ellis, 1977).

The pattern of polypeptides synthesized by intact washed chloroplasts incubated in the light is shown in Figure 25. This pattern can be compared with that shown in Figure 24, which was taken from a separate experiment. The two patterns are very


LSU
PEAK D
A
B
C
D
E

similar. These chloroplast incubations incorporate isotope into LSU, peak D and the α and β CF_1 subunits, as well as into many unidentified, less heavily-labelled polypeptides. When chloroplasts are lysed, and fractionated before incubation, the pattern of incorporation into stromal polypeptides is that shown in Figure 25, track B. The most heavily labelled band is LSU although some incorporation into the α and β subunits of CF_1 is visible. These two subunits are heavily labelled in the membrane fraction. The CF_1 portion of the chloroplast ATP synthase complex, although a thylakoid peptide, is loosely bound to the membrane. Up to 50% of the CF_1 polypeptides can be removed by 1 mM EDTA or conditions of low ionic strength (Nelson, 1976). The synthesis of the α and β CF_1 polypeptides is therefore not necessarily expected to take place exclusively on membrane-bound ribosomes, as might be predicted for an intrinsic membrane polypeptide. However, since CF_1 is readily removed from thylakoids, the presence of labelled α and β polypeptides in the soluble fraction could also be an artefact of the fractionation procedure.

The major difference between the products of protein synthesis in intact and lysed chloroplasts is the synthesis of the intrinsic membrane polypeptide, peak D. Peak D is synthesized in intact isolated chloroplasts (Figure 25, track A), but is not labelled in either the soluble fraction (track B) or the membrane fraction (track C) of lysed chloroplasts. A similar situation has been described for lysed pea chloroplast preparations (Ellis, 1977) where soluble ribosomes synthesize LSU, but no visible peak D is synthesized on either soluble or membrane-bound ribosomes. The background of radioactivity observed in the membrane polypeptide patterns on autoradiographs in both these studies may be

indicative of incomplete synthesis of peak D. Bottomley et al (1974), however, were able to obtain incorporation of isotope into the 36 000 M.wt. polypeptide in broken spinach chloroplasts, although they did not fractionate the chloroplasts. There is no simple explanation for the discrepancy between these studies in view of the following points:-

1. the three studies discussed used the same incubation buffer;
2. fractionation of the chloroplasts before incubation is not the cause of loss of synthesis of peak D. In Figure 25 (track D), intact spinach chloroplasts incubated in the light synthesize peak D, whereas the same preparation incubated in the dark with ATP and GTP after lysis makes no peak D;
3. contaminating whole cells are unlikely to be the cause of the observations of Bottomley et al (1974) because cycloheximide did not inhibit incorporation of isotope into broken chloroplast preparations (see their Table IV).

It is more likely that the exact conditions of isolation and preparation of chloroplasts affects the synthesis of some polypeptides more than others. It is not known whether re-initiation of protein synthesis takes place in lysed chloroplast preparations; if the lack of a major stained band of the mobility of peak D is a reflection of rapid synthesis and degradation of the complete polypeptide by (specific ?) proteolysis, then the non-appearance of peak D could be explained by a lack of reinitiation. This question will only be resolved by a closer study of the mechanism of protein synthesis in isolated, intact and lysed chloroplasts.

In summary, it is possible to isolate intact chloroplasts from spinach primary leaves which incorporate [^{35}S] methionine into polypeptides on 70S ribosomes, and use light as the major

energy source. The characteristics of the system are similar to previously published studies on pea and spinach. However, the activity of such chloroplast preparations is disappointing when compared to that found for pea chloroplasts. The incorporation of isotope into polypeptides in the dark is probably more obvious in this system than in a more active system such as pea because the incorporation in the light relative to the dark control is so poor. The dark background of products is puzzling because it is found in chloroplasts isolated from both young and old tissue, and represents a partial spectrum of chloroplast-synthesized polypeptides. A broad,diffuse band is visible in the SSU region of gels of in vitro-synthesized chloroplast polypeptides which does not represent this polypeptide because its synthesis is insensitive to cyclo-heximide (see Figure 24, tracks J and K). The dark background of incorporation is unlikely to be due to contaminating bacterial or cytoplasmic protein synthesis because the polypeptide pattern synthesized is similar to that synthesized in isolated chloro-plasts. It may be a consequence of tissue growth conditions. The conditions of growth, as discussed in Section III.1, are an important factor in determining the rate of accumulation of chloroplast components during leaf development. The compost-grown plants, which grow more slowly than hydroponically-grown plants, yield less active chloroplasts, possibly because of some limitation of their growth conditions. However, important parameters of the growth conditions of both types of plant are the light intensity (10 000 lux) and the photoperiod (8 hours). These two parameters were fixed at the values stated because they allowed the growth of tissue without starch accumulation, an important factor in the successful isolation of intact

chloroplasts. The growth of plants under conditions which prevent starch formation may result in a lower protein-synthetic activity in the isolated chloroplasts. Perhaps the successful use of pea chloroplasts in _in vitro_ protein synthesis is a reflection of the rapid growth of these plants under low light intensity (2 000 lux) and long photoperiod (12 hours) without starch accumulation.

Despite these problems, the chloroplasts isolated from spinach primary leaves do incorporate [^{35}S] methionine into LSU and peak D, and can therefore be used to estimate the ratio of incorporation into these polypeptides _in vitro_ during leaf development.

B. _Estimation of the ratio of incorporation of [^{35}S] methionine into LSU and peak D_

Having established that isolated chloroplasts prepared from spinach primary leaves incorporate [^{35}S] methionine into the two major chloramphenicol-sensitive polypeptides labelled _in vivo_, it is possible to extend the measurements made in Section III.2 to isolated chloroplasts. However, it is important to establish first whether a change in the amount of incorporation of isotope into LSU and peak D can be observed on autoradiographs of gels of the products of protein synthesis in chloroplasts isolated from developing leaves. Figure 26 shows the pattern of polypeptides labelled in isolated chloroplasts prepared from 12 day leaves (tracks B and C) and 20 day leaves (tracks F, G, H and I), and the stained pattern of polypeptides obtained from the two ages of tissue (tracks D and J). The following points can be seen in this figure:-

1. the patterns of stained bands revealed by Coomassie Blue dye in 12 day (track D) and 20 day (track J) isolated chloroplasts

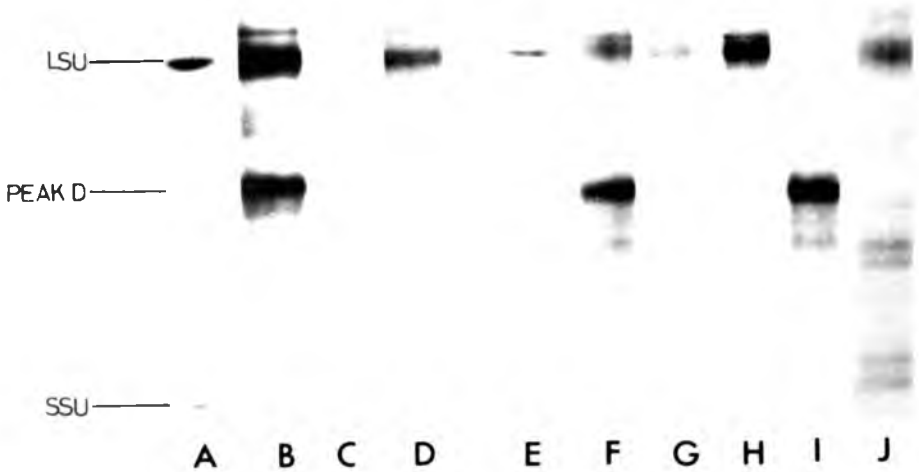

LSU
PEAK D
SSU
A
B
C
D
E
F
G
H
I
J

Figure 26

Products of protein synthesis in intact chloroplasts prepared from two ages of spinach primary leaf

Chloroplasts were prepared from 12 day and 20 day leaves as described in Section II 2D(i). Aliquots (1.0 ml) of chloroplast suspension containing 240 µg chlorophyll and 100 µCi [^{35}S]methionine were incubated as described in Section II 2D(ii). Products were analyzed on 7.5-15% linear polyacrylamide gradient Chua gels containing SDS (Section II 2F(i)b), dried down and autoradiographed (Section II 2K). Tracks B, C and D were loaded with samples equivalent to 2.5 µg chlorophyll; tracks F, G, H, I and J were loaded with samples equivalent to 4 µg chlorophyll. Tracks A, B and C were autoradiographed for 7 days; tracks F, G, H and I were autoradiographed for 3 days.

A. [^{14}C]-labelled spinach Fraction I protein marker
B. 12 day leaf chloroplasts incubated in the light
C. 12 day leaf chloroplasts incubated in the dark
D. Pattern of Coomassie Blue-stained bands from Track B
E. As for Track A
F. 20 day leaf chloroplasts incubated in the light
G. 20 day leaf chloroplasts incubated in the dark
H. Soluble polypeptides from Track F
I. Membrane-bound polypeptides from Track F
J. Pattern of Coomassie Blue-stained bands from Track F

are virtually identical. This observation extends similar conclusions made in Section III.2, where extracts of whole leaves were analyzed. The similarity in staining pattern between extracts of leaves and isolated chloroplasts indicates that the majority of leaf proteins are located in the chloroplast fraction;

2. chloroplasts isolated from 20 day tissue synthesize LSU as a soluble polypeptide (track H) and peak D as an insoluble polypeptide (track I). The α and β suunits of CF_1 are present in both soluble and membrane fractions, although they are more heavily labelled in the soluble fraction (track H). This fractionation pattern is similar to that found for whole leaf polypeptides labelled *in vivo* (Figure 19);

3. the qualitative patterns of polypeptides labelled with [^{35}S]methionine in chloroplasts from 12 day leaves (track B) and chloroplasts from 20 day leaves (track F) are similar on a gross level. The major changes visible in incorporation are quantitative. The incorporation of isotope into LSU relative to peak D at 12 days is less than after 20 days;

4. the apparent incorporation of isotope in the dark in intact chloroplasts is visible in chloroplasts isolated from tissue after 12 days (track C) and 20 days (track G).

5. the β polypeptide of spinach CF_1 (M.wt. 57 000, Binder *et al*, 1978) is resolved from LSU (M. wt. 55 800, Rutner, 1970) on the gel system employed. In spinach, resolution of these two polypeptides is possible because the molecular weights are sufficiently disparate; in pea, the β polypeptide co-electrophoreses with LSU.

The data shown in Figure 26 indicate that there is a change in incorporation of isotope into LSU and peak D relative to each

other. In order to quantitate this change, autoradiographs of polyacrylamide gels of polypeptides labelled in the light in chloroplasts isolated from developing spinach leaves were scanned with a Joyce-Loebl scanning densitometer. Peak height was used as a measure of changes in incorporation into LSU relative to peak D as described for *in vivo* measurements (Section III.2). Total chloroplast polypeptide tracks were used for quantitation rather than soluble and membrane fractions because the α and β CF_1 polypeptides were resolved from LSU without the need for fractionation. Since there is incorporation into some chloroplast polypeptides in the dark, the dark background scan was sub tracted from the appropriate light scan to correct for this extra incorporation. This subtraction also corrects for any lysis of chloroplasts during incubation resulting in synthesis of a partial spectrum of polypeptides. The dark background observed did not contain all the polypeptides labelled in the light; LSU is labelled in the dark, whereas peak D is not, and without correction the ratios of incorporation into LSU realtive to peak D would be prejudiced in favour of LSU.

Table IV shows the incorporation of [^{35}S] methionine into LSU relative to peak D in isolated chloroplasts prepared from developing leaves. Chloroplasts were isolated from tissue ranging from 12 days old to 35 days old, a time period which, in Section III.1, was shown to include the phase of maximum accumulation of chlorophyll and Fraction I protein. From the data in Table IV, it can be seen that while leaf-length increases six-fold, there is a four-fold decrease in the incorporation of isotope into LSU relative to peak D in isolated chloroplasts over a period of 23 days. These date indicate that

Day after germination	Leaf length (mm)	Ratio of incorporation of [^{35}S] methionine into LSU:peak D
12	8.5	0.76, 0.70
12	9.0	0.79, 0.83
17	23.0	0.51, 0.58
17	34.4	0.34, 0.55
20	26.4	0.30, 0.36
24	35.9	0.30, 0.29
24	50.0	0.33, 0.32
35	49.6	0.14, 0.16

Table IV

Calculated ratios of [^{35}S] methionine incorporation into LSU relative to peak D in chloroplasts isolated from developing hydroponically-grown spinach primary leaves.

Autoradiographs of the products of protein synthesis by isolated chloroplasts incubated with [^{35}S] methionine, as described in Figure 26, were scanned with a Joyce-Loebl scanning densitometer. Peak heights were used to calculate the ratio of incorporation of isotope into LSU relative to peak D for 2 loadings at each time point. Data are taken from several experiments.

in hydroponically-grown plants, the ratio of incorporation of isotope into LSU relative to peak D decreases both *in vivo* (Figure 22) and *in vitro* (Table IV) in a similar fashion, when compared on the basis of either leaf length or time after germination. What does differ between the two situations so far examined in this study is the absolute value of the ratios, and this point will be discussed in Section IV. However, despite this difference in the absolute ratios of incorporation of isotope into LSU relative to peak D, it is clear that whatever factors are responsible for the net decrease in the ratio *in vivo* are also operating in isolated chloroplasts.

C. Discussion

Two main points emerge from the data presented in this section:-

1. isolated spinach primary leaf chloroplasts heavily label two polypeptides, LSU and peak D, when incubated in the light with [^{35}S]methionine. The labelling of these polypeptides *in vivo* was found to be chloramphenicol-sensitive and cycloheximide-insensitive (Section III.2);

2. the ratio of incorporation of [^{35}S]methionine into LSU relative to peak D in chloroplasts isolated from developing spinach primary leaves follows a similar pattern to that observed *in vivo*.

These points will now be discussed in more detail, with reference to related studies in the literature.

Since the first analysis of the soluble products of protein synthesis in isolated pea chloroplasts (Blair and Ellis, 1973), a number of other species have been investigated. In pea, the major soluble product of chloroplast protein synthesis present in

a 150 000 g supernatant fraction was LSU, its identity being confirmed by tryptic peptide mapping. The major thylakoid polypeptide labelled in isolated pea chloroplasts was a 32 000 M. wt. polypeptide which, although it was heavily labelled, did not correspond to any stained band on SDS-polyacrylamide gels (Eaglesham and Ellis, 1974). This polypeptide, which is still of unknown function, does not correspond in electrophoretic mobility to cytochrome f, any CF_1 polypeptides or a CPI or CPII polypeptide. Bottomley et al (1974) analysed total and soluble products of protein synthesis in isolated spinach chloroplasts and obtained patterns of incorporation very similar to those described in pea, including the synthesis of a heavily-labelled, non-stainable polypeptide of 36 000 M. wt..Morgenthaler and Mendiola-Morgenthaler (1976) have also examined the synthesis of soluble, thylakoid and envelope polypeptides in a highly purified spinach chloroplast preparation. On discontinuous sucrose gradients, 50% of the isotope incorporated into such intact, purified chloroplasts fractionates into the thylakoids, compared with 29% in the soluble and only 0.2% in the envelope fractions. Thus, in whole chloroplast preparations, the envelope polypeptides do not give rise to heavily-labelled bands on SDS-polyacrylamide gels. The patterns of incorporation of isotope into soluble and thylakoid polypeptides were similar to those observed in pea by Blair and Ellis (1973) and Eaglesham and Ellis (1974) where crude plastid preparations were employed. However, Joy and Ellis (1975) resolved two major peaks of incorporation (38 000 and 28 000 M. wt. respectively) into envelope polypeptides purified from isolated, labelled chloroplasts from pea, whereas Morgenthaler and Mendiola-Morgenthaler (1976) observed only one major peak

(50 000 M. wt.). In the case of envelope fractions, the problems of contamination of the relatively minor envelope fraction by more abundant thylakoid polypeptides have not been completely resolved, so it is not clear whether spinach differs from pea in its envelope polypeptides or not.

Although the bulk of work on protein synthesis in isolated chloroplasts has been carried out using pea or spinach as starting tissue, the pattern of polypeptides synthesized by other tissues is similar to these two plants. Barley chloroplasts synthesize polypeptides having the electrophoretic mobilities of LSU and peak D on SDS polyacrylamide gels. Young maize leaf chloroplasts synthesize only peak D,which is consistent with the observation that most of the chloroplasts derive from mesophyll cells which lack Fraction I protein (Ellis, 1977). This observation that maize mesophyll chloroplasts do not label LSU in vitro has recently been confirmed by Link et al (1978), who have demonstrated that although these chloroplasts contain the LSU gene, they contain no hybridizable or translatable LSU mRNA. It appears also that algal chloroplast protein synthetic products resemble those described in higher plant chloroplasts. Vasconcelos (1976), using silica-sol purified chloroplasts from Euglena gracilis, has shown that the major soluble product of chloroplast protein synthesis is LSU. However, there are some differences between the pattern of incorporation into algal and higher plant thylakoid polypeptides, for example the relative incorporation into 42 000 and 31 000 M. wt. polypeptides. Vasconcelos speculates that this difference may reflect a significant difference in their patterns of polypeptide synthesis. It seems likely, therefore,

that although only a small number of plant tissues have so far proved suitable for the preparation of isolated chloroplasts active in protein synthesis, new species will probably be found to synthesize a spectrum of polypeptides broadly similar to those described above. However, it may not be possible to extrapolate from one species to another with respect to the synthesis of specific polypeptides since the mobilities and amounts synthesized vary considerably.

As was discussed in Section I, the number of polypeptides detectable as being synthesized in isolated chloroplasts increases as better resolution is obtained on SDS polyacrylamide gels. Mendiola-Morgenthaler et al (1976) treated isolated spinach chloroplasts with EDTA, using the procedure of Strotmann et al (1973), and showed, contrary to early studies by Eaglesham and Ellis (1974), the α, β and possibly ε subunits of CF_1 are synthesized in isolated spinach chloroplasts. The authenticity of the polypeptides having the mobilities of the α and β polypeptides synthesized in isolated chloroplasts from spinach has been confirmed by Grebanier et al (1978), using the one-dimensional mapping procedure of Cleveland et al (1977). Ellis has confirmed that α, β and ε polypeptides are synthesized in isolated pea chloroplasts, and attributes earlier failure to detect this to the fact that together these subunits contain only 1% of the label incorporated into membrane protein. There is now evidence of the synthesis of other relatively minor chloroplast components in isolated chloroplasts. Cytochrome f is synthesized by isolated pea chloroplasts (Docherty and Gray, 1979) and cytochrome b_{559} is synthesized in isolated spinach chloroplasts (Zielinski and Price, 1979). The problems in the area of identification of chloroplast polypeptides are now ones

of quantity, since the amount of incorporation of isotope into a minor membrane component such as cytochrome f when compared to LSU is very low.

The ultimate fate of polypeptides synthesized in isolated chloroplasts has been discussed in Section I. Grebanier et al (1978) have recently reported that a 32 000 M. wt. polypeptide, heavily labelled in vivo, is made as a 34 500 precursor in maize chloroplasts, but is not processed in vitro. The newly synthesized precursor is not integrated into thylakoids in isolated chloroplasts, since it is wholly susceptible to protease digestion, whilst a 19 000 M. wt. fragment of the 32 000 M. wt. polypeptide is resistant to such digestion. The heavy labelling of this polypeptide and its mobility on SDS polyacrylamide gels suggests that it may be analogous to peak D in in pea (Eaglesham and Ellis, 1974) and the 36 000 M. wt. polypeptide in spinach (Bottomley et al , 1974). However, in maize this polypeptide is present in stainable amounts implying that it may not turn over as observed in pea and spinach. In the present study, the gel system used would not resolve the precursor and product, but synthesis of peak D as a precursor in spinach is unlikely because it fractionates entirely as a membrane-bound polypeptide. This is also true in pea since Eaglesham and Ellis (1974) found that peak D was specifically a thylakoid polypeptide and this polypeptide was not present in the post-ribosomal supernatant analyzed by Blair and Ellis (1973). If the polypeptide studied by Grebanier et al (1978) does correspond to peak D, then the mechanism of its synthesis may differ from plant to plant.

The inital aim of this section was to compare the change in ratio of incorporation of [^{35}S] methionine into LSU relative

to peak D during spinach primary leaf development *in vivo* with that in isolated chloroplasts. The similarity between the two situations with respect to the decreasing ratio indicates that isolated chloroplasts intrinsically determine the ratio of these two polypeptides since short-term removal from the cell does not affect the change in this ratio. This does not imply that there is no cytoplasmic involvement, but rather that isolated chloroplasts contain the mechanism of control. The obvious testable levels of control inside the chloroplast are either at the level of transcription of the mRNA's for these polypeptides, as is the case for LSU in maize mesophyll cells (Link *et al* , 1978), or at the level of translation. There are readily available methods for assay of translatable mRNA in cell-free protein synthesizing systems, and this approach should yield further information about the control of gene expression in spinach primary leaf chloroplast development.

III.4 IN VITRO SYNTHESIS OF CHLOROPLAST POLYPEPTIDES IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM DIRECTED BY TOTAL CHLOROPLAST RNA

A. Characteristics of the nuclease-treated cell-free system

The nuclease-treated reticulocyte lysate cell-free system (Pelham and Jackson, 1976) is an ideal system for use in the study of the synthesis of polypeptides directed by exogenous RNA. As a result of nuclease treatment, the endogenous background synthesis is low compared to, for example, the wheat germ cell-free system (Roberts and Patterson, 1973). The extract is capable of synthesizing high molecular weight polypeptides with a low proportion of apparently incomplete cha ins (Pelham and Jackson, 1976), when compared to a wheat germ extract (Tse and Taylor, 1971). Nuclease-treated reticulocyte lysate extracts have been used to translate mRNA's with fidelity from a wide range of sources, for example:-

α-crystallin (Asselbergs et al , 1978);

mouse interferon (Lebleu et al , 1978);

cowpea mosaic virus (Pelham and Stuik, 1976);

phenylalanine ammonia lyase (Ragg et al , 1977);

turnip yellow mosaic virus (Benicourt et al , 1978).

For these reasons, the nuclease-treated reticulocyte lysate was used to translate chloroplast mRNA and to estimate amounts of translatable mRNA.

Table V shows the characteristics of incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble material in a reticulocyte lysate extract. The nuclease-treated system was found to be almost totally dependent on added mRNA. Any endogenous incorporation was found to result mainly from incorporation into globin (see, for example, Fig. 28). When

	% incorporation
Complete	100
- added RNA	0.8
+ cycloheximide (100 µg/ml)	0.3
+ chloramphenicol (100 µg/ml)	110
- creatine phosphate	0

Table V Characteristics of incorporation of [^{35}S] methionine into trichloroacetic acid-insoluble material by the reticulocyte lysate cell-free protein-synthesizing system.

Reticulocyte lysate reaction mixtures were set up as described in Section II 2E(ii)a. The complete assay tube in each case contained 75% (v/v) nuclease-treated lysate, 10 mM creatine phosphate, amino acids (except methionine) at the frequency found in globin, 75 mM potassium chloride, 0.5 mM magnesium acetate, 5 µCi [^{35}S] methionine and 5 µg TMV RNA in a final volume of 20 µl. Other additions are indicated in the table. After incubation at 30° C for 60 minutes, 2 µl aliquots were assayed for trichloroacetic acid-insoluble incorporation (Section II 2E(ii)a), and the values obtained expressed as a percentage of the complete incubation value. The complete mixture incorporated 151 600 cpm/2 µl aliquot.

creatine phosphate is omitted from the incubation mixture, there is little incorporation indicating that the lysate is dependent on an added energy source. The total inhibition of incorporation by cycloheximide, but not by chloramphenicol, indicates that protein synthesis is taking place on 80S ribosomes.

Figure 27 shows the time course of incorporation in a reticulocyte lysate extract. In the absence of added RNA, there is little incorporation of [^{35}S] methionine into trichloroacetic acid-insoluble material, even after 2½ hours. When TMV RNA is added, there is a short lag-phase, and then incorporation is linear for 20-30 minutes. After 60 minutes, there is some loss of trichloroacetic acid-insoluble incorporation, amounting at 150 minutes to about 15% of the maximum incorporation. This may be indicative of some proteolytic activity in the reticulocyte lysate, so subsequent incubations were terminated after 60 minutes.

Figure 28 shows the polypeptides synthesized in a reticulocyte lysate system directed by chloroplast RNA at increasing times of incubation. The lysate synthesizes a pattern of polypeptides which is qualitatively similar to that synthesized in isolated chloroplasts (see, for example, Fig. 26). However, it is clear that peak D is not a major labelled polypeptide in this reticulocyte lysate system, as it is in isolated chloroplasts (Section III 3). There is also incorporation into a polypeptide with an apparent molecular weight of about 20 000. It is not clear whether this polypeptide results from a small amount of contaminating p20 mRNA (Section I 2C) or is a product of the translation of chloroplast mRNA. The former possibility seems unlikely for the following reasons:-

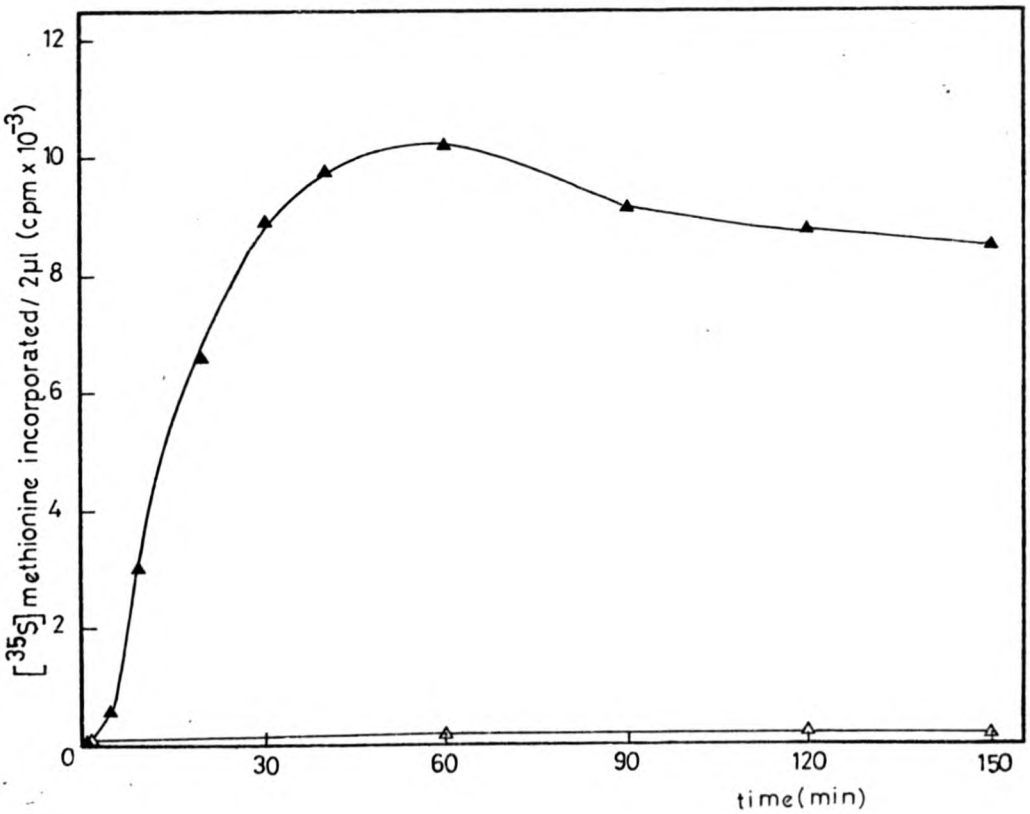

12
10
8
6
4
2
0
30
60
90
120
150
time(min)
[35S] methionine incorporated/ 2µl (cpm x 10-3)

Figure 27 Time course of incorporation of [^{35}S] methionine in the reticulocyte lysate cell-free protein-synthesizing system.

A reticulocyte lysate assay was performed under the conditions described in Table III. Incubation was at 30° C, and 2 µl aliquots were removed at the indicated times, and analyzed for trichloroacetic acid-insoluble incorporation (Section II 2E(ii)a).

△——△ no added RNA

▲——▲ + 5 µg TMV RNA

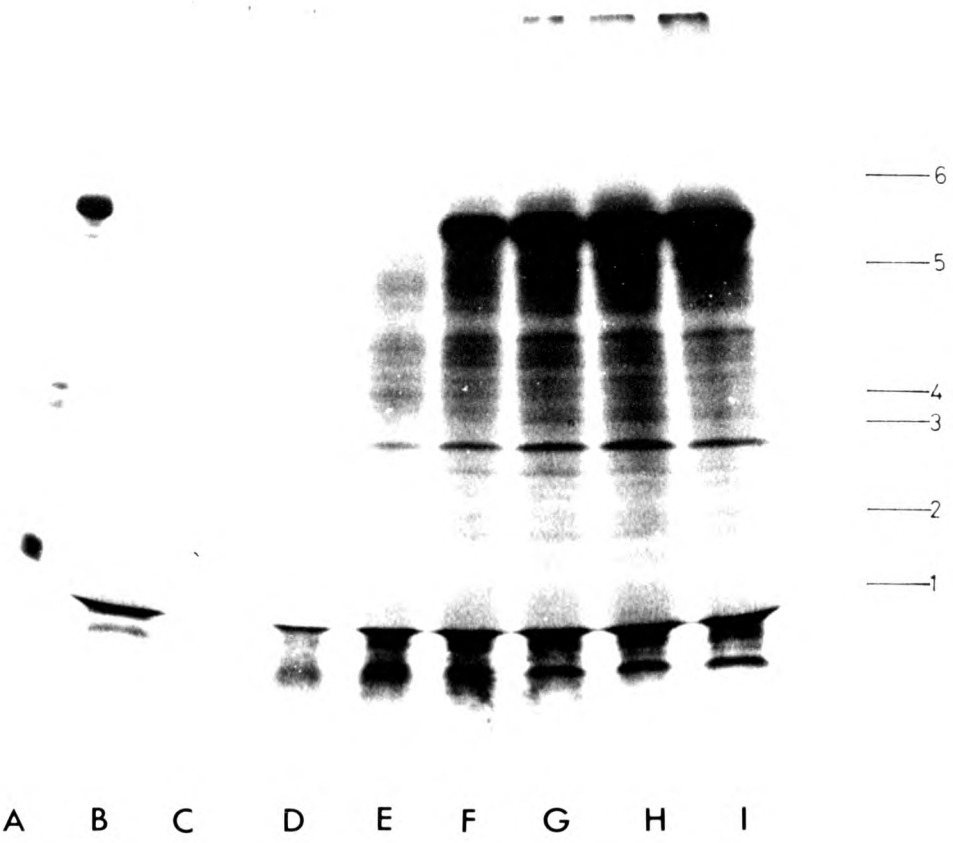

6
5
4
3
2
1
A
B
C
D
E
F
G
H
I

Figure 28 Products of a time course of protein synthesis in a reticulocyte lysate cell-free system programmed with chloroplast RNA.

A reticulocyte lysate assay was performed under the conditions described in Section II 2E(ii)a. Each incubation tube contained 75% (v/v) nuclease-treated lysate, 10 mM creatine phosphate, amino acids at the frequency found in globin (except methionine), 75 mM potassium chloride, 0.5 mM magnesium acetate, 5 µCi [^{35}S]-methionine and, where indicated, 7.5 µg spinach chloroplast RNA. The tubes were incubated for the indicated times. Mixtures were prepared for electrophoresis as described in Section II 2E(ii)a, and analyzed on a Laemmli SDS gel (Section II 2F(i)b) containing a 10-30% linear polyacrylamide gradient. The dried gel was autoradiographed for 1 week.

A. [^{14}C]-labelled spinach Fraction I protein.

B. No added RNA after 60 minutes incubation.

Tubes C-I contained chloroplast RNA and were incubated for C, 0 min; D, 5 min; E, 10 min; F, 20 min; G, 30 min; H, 40 min; I, 60 min.

The molecular weight markers are listed below.

1. Haemoglobin (15 500)
2. Myoglobin (17 200)
3. Trypsin (23 000)
4. Chymotrypsin (25 000)
5. Ovalbumin (43 000)
6. BSA (68 000)

6
5
4
3
2
1

1. isolated chloroplasts synthesize a polypeptide of this mobility;
2. there is no obvious cytoplasmic rRNA contamination of the chloroplast RNA preparations (Section III 4C).

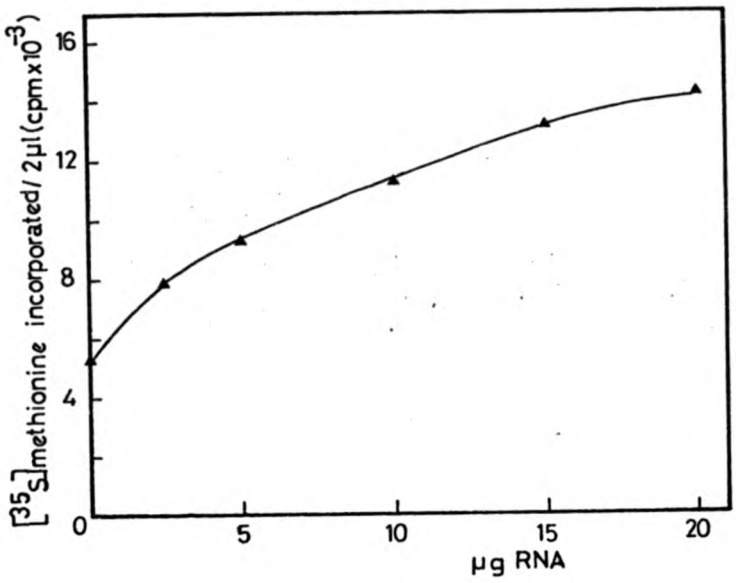
To resolve this point it would be necessary to compare the tryptic peptide maps for P20 and the reticulocyte lysate polypeptide of the same mobility. A comparable pattern of polypeptides to that shown in Fig. 26 has been observed in a reticulocyte lysate programmed with maize chloroplast RNA (Coen et al , 1978). However, the endogenous pattern of incorporation in the maize system includes a heavily-labelled polypeptide with a slightly faster mobility than LSU, which is absent in the system used in the present study. It is also clear that LSU mRNA from maize is not translated as efficiently as the mRNA for the 32 000 M. wt. thylakoid polypeptide in their system.

The appearance of chloroplast polypeptides in Figure 28 follows a similar time course to that observed by Hunter et al (1977) using TMV RNA in a wheat germ system. Low molecular weight polypeptide bands, e.g. 20 000 M. wt. appear early on and then reach a plateau of intensity, suggesting that there is a limited period of initiation followed by polysome run-off. This has also been observed in extracts of L cells (Boime and Leder, 1972) and Krebs II ascites cells (Kerr et al , 1972). Using the method of Matthews and Osborn (1964), it is possible to obtain an estimate of the rate of chain elongation. If it is assumed that the average molecular weight of an amino acid is 115, and that the first evidence of a band of the mobility of LSU is at 10 minutes, then the rate of polypeptide chain elongation is 48 amino acids/minute. Values of 100 amino acids/

minute have been measured in wheat germ extracts (Hunter et al., 1977) and 25 amino acids/minute in Krebs II ascites extracts (Boime and Leder, 1972).

Figure 29 shows the effect of RNA concentration on incorporation of [^{35}S] methionine into trichloroacetic acid-insoluble material by the reticulocyte lysate cell-free system. The addition of increasing amounts of chloroplast RNA to the assay system results in an increase in incorporation (Fig. 29A). The increase in incorporation is linear between 5 and 15 µg added RNA. It is clear that, although a linear increase in incorporation results when increasing amounts of chloroplast RNA are added to the system, the increased incorporation is not proportional to the amount of RNA added. Addition of increasing amounts of TMV RNA to this sytem results in a sharp increase in incorporation until 4 µg RNA per assay, and then a decrease in incorporation (Fig. 29B). This change in incorporation presumably reflects the effect of adding saturating amounts of mRNA to the cell-free system. The TMV RNA preparation contains no ribosomal RNA and so would be expected to saturate the system more rapidly than total chloroplast RNA. It is clear from Figure 29 that chloroplast RNA is not as efficient a template for protein synthesis in the reticulocyte lysate as TMV RNA is, and this must be due, in part, to the large amounts of rRNA in the total RNA preparations.

The products of protein synthesis in the reticulocyte lysate directed by increasing amounts of chloroplast total RNA and TMV RNA are shown in Figure 30. Addition of increasing amounts of chloroplast RNA results in increased synthesis of many polypeptides, including those with the mobility of LSU, the α and β subunits of CF_1 and the 20 000 M. wt. polypeptide (Figure 30, tracks B-F). TMV RNA (at 1, 2 and 4 µg/20 µl) directs the synthesis of several


[35S]methionine incorporated/ 2µl (cpm x 10-3)
16
12
8
4
0
5
10
15
20
µg RNA

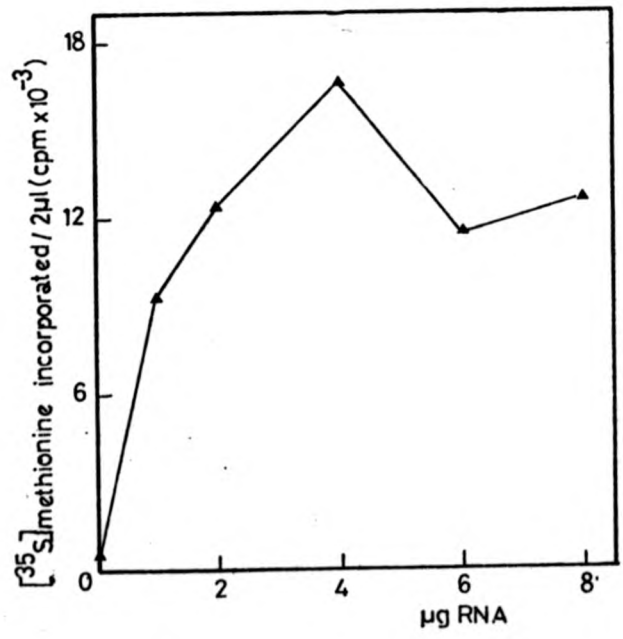

[35S]methionine incorporated/ 2µl (cpm x 10-3)
18
12
6
0
2
4
6
8
µg RNA

Figure 29 The effect of increasing RNA concentration on [^{35}S] methionine incorporation in a reticulocyte lysate cell-free system.

A reticulocyte lysate assay was performed under the conditions described in Table III and RNA added at the indicated amounts. Incubation was at 30° C for 60 minutes. Aliquots (2 µl) were removed at zero time and after 60 minutes, and analyzed for hot trichloroacetic acid-insoluble incorporation (Section II 2E(ii)a). The incorporation at zero time was subtracted from that obtained after 60 minutes incubation.

A. Spinach leaf chloroplast RNA

B. Tobacco mosaic virus RNA

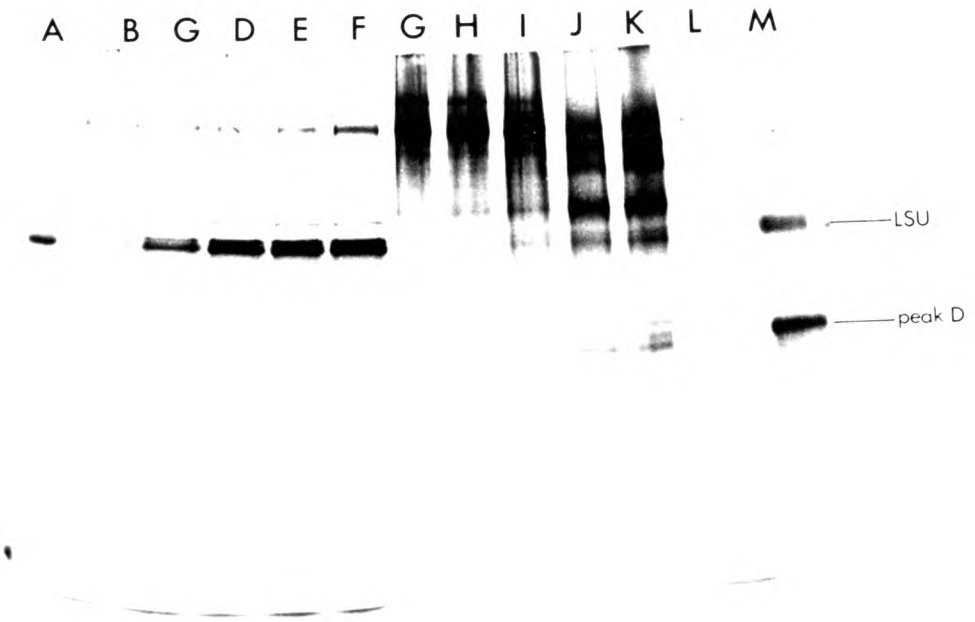

A B G D E F G H I J K L M
LSU
peak D

Figure 30 Polyacrylamide slab gel electrophoresis of reticulocyte lysate products synthesized in response to increasing RNA concentration.

Reticulocyte lysate assay incubations (20 µl) were set up as described in Section II 2E(ii)a. The tubes were incubated for 60 minutes at 30° C and the mixtures prepared for electrophoresis by the method described in Section II 2E(ii)b. Equal portions of the incubation mixtures were analyzed on a Laemmli SDS gel (Section II 2F(i)b) containing a 10-30% polyacrylamide gradient. The gel was dried down and autoradiographed (Section II 2K) for 9 days.

—LSU

—peak D

A. [^{14}C]-labelled spinach Fraction I protein

B. 2.5 µg chloroplast RNA

C. 5 µg chloroplast RNA

D. 10 µg chloroplast RNA

E. 15 µg chloroplast RNA

F. 20 µg chloroplast RNA

G. 1 µg TMV RNA

H. 2 µg TMV RNA

I. 4 µg TMV RNA

J. 6 µg TMV RNA

K. 8 µg TMV RNA

L. no added RNA

M. [^{35}S]methionine-labelled products of protein synthesis in isolated spinach chloroplasts

high molecular weight polypeptides, giving rise to a pattern similar to that observed by Pelham and Jackson (1976). At higher TMV RNA concentrations (6 and 8 µg/20 µl incubation), the products are of lower molecular weight than at low RNA concentration. This phenomenon has also been observed by Pelham and Jackson (1976), and is reversed by the addition of tRNA. At saturating RNA concentrations, another factor involved in determining the spectrum of products is the relative translatability of mRNA s in the assay mixture. Lodish (1974) has analysed the effect of partial inhibition of the various steps in protein synthesis on the relative rates of translation of various mRNA's. Under conditions of competition for initiation factors, some mRNA's are translated more efficiently than others and the products of protein synthesis may not reflect the actual mRNA population (Section I 4). This situation arises during conditions of RNA saturation and may account in part for the change in the synthesis of TMV polypeptides. However, more importantly, in the range of chloroplast RNA concentrations tested, there was no obvious change in the spectrum of polypeptides synthesized. This observation lends support to the hypothesis that the products of chloroplast RNA-directed protein synthesis in the reticulocyte lysate system reflect the amounts of translatable mRNA present.

The effect of salt concentration on trichloroacetic acid-insoluble incorporation in a reticulocyte lysate directed by chloroplast and TMV RNA is shown in Figure 31. The potassium ion optimum for isotope incorporation into chloroplast polypeptides is not pronounced, but occurs at about 75 mM (Fig. 31C). This differs from the optimum potassium ion concentration for TMV RNA translation which is 150 mM (Fig. 31A). The optimum magnesium ion

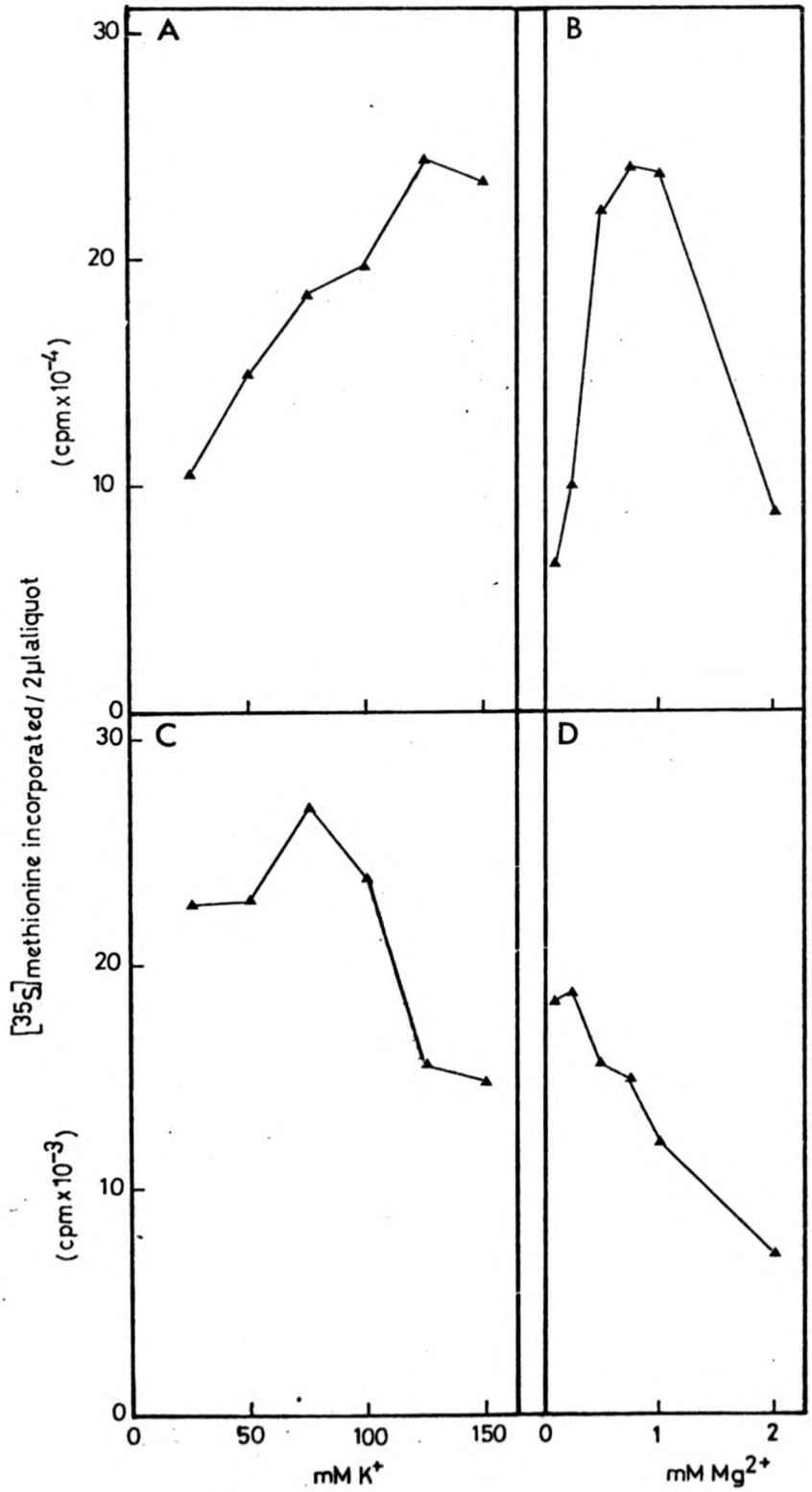

A
B
C
D
30
20
10
0
(cpm x 10⁻⁴)
(cpm x 10⁻³)
[35S]methionine incorporated / 2µl aliquot
0
50
100
150
mM K+
0
1
2
mM Mg2+

Figure 31 The effect of potassium and magnesium ion concentration on incorporation of [^{35}S]-methionine by a reticulocyte lysate extract.

Incubations containing 75% (v/v) nuclease-treated lysate, 10 mM creatine phosphate amino acids at the frequency found in globin (except methionine), 5 µCi [^{35}S]methionine and the RNA and salt concentrations indicated below were incubated for 60 minutes at 30° C (Section II 2E(ii)a). Aliquots (2 µl) were analyzed for hot trichloroacetic acid-insoluble incorporation by the method in Section II 2E(i)b. The incorporation at zero time was subtracted from the values obtained after 60 minutes.

A. 5 µg TMV RNA, 0.5 mM magnesium acetate and the potassium chloride concentrations indicated.

B. 5 µg TMV RNA, 75 mM potassium chloride and the magnesium acetate concentrations indicated.

C. 10 µg chloroplast RNA, 0.5 mM magnesium acetate and the potassium chloride concentrations indicated.

D. 10 µg chloroplast RNA, 75 mM potassium chloride and the magnesium acetate concentrations indicated.

concentrations for translation of TMV and chloroplast mRNA's are 0.75 mM and 0.5 mM respectively. These values are probably underestimates of the true optimal salt concentrations since unfractionated reticulocyte lysate also contains endogenous salts. The salt concentration optima measured for incorporation into TMV polypeptides are similar to those found by Pelham and Jackson (1976).

Figure 33 shows the polypeptides synthesized in response to added chloroplast mRNA with increasing potassium ion concentration. The peak of synthesis of LSU and peak D occurs in 75 mM KCl which is also the optimum for total incorporation. At 150 mM KCl, the highest salt concentration tested, there is a greatly reduced synthesis of chloroplast polypeptides. This finding is consistent with the observation that increased potassium ion concentrations inhibit initiation (Hunter et al , 1977). Magnesium ion concentration does not have such a marked effect on the spectrum of products synthesized in a reticulocyte lysate extract programmed with chloroplast RNA (Figure 32). The optimum concentration of magnesium acetate for synthesis of LSU and peak D is about 0.5 mM, and since this concentration was also the optimum for trichloroacetic acid-insoluble incorporation, it was used in all subsequent assays.

B. Partial proteolytic digestion of chloroplast polypeptides

The data presented so far in this section indicate that the reticulocyte lysate translates chloroplast mRNA to yield polypeptides which co-electrophorese with those synthesized in isolated chloroplasts. The system responds to increasing amounts of added chloroplast RNA in a linear fashion within the range of 5-15 µg per assay, which allows its use as a quantitative assay for translatable mRNA. However, before this system can be used to

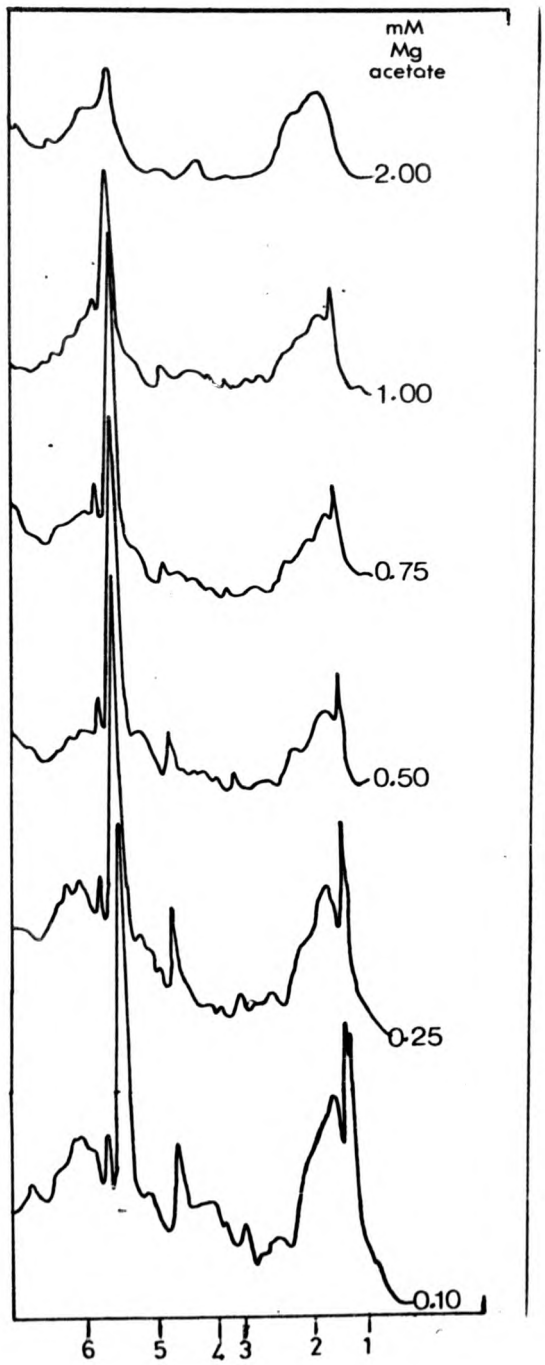

mM
Mg
acetate
2.00
1.00
0.75
0.50
0.25
0.10
6
5
4
3
2
1

Figure 32 Products of protein synthesis in a reticulocyte lysate programmed with chloroplast RNA with increasing magnesium ion concentration.

Reticulocyte lysate incubations (20 µl) were set up as described in Section II 2E(i)a and contained 10 µg chloroplast RNA, 75 mM potassium chloride and the magnesium acetate concentrations indicated. Incubation was at 30° C for 60 minutes. Equal portions of each incubation were prepared for electrophoresis by the method set out in Section II 2E(i)b and analysed by electrophoresis on a 10-30% polyacrylamide gradient Laemmli gel containing SDS (Section II 2F(i)b). The gel was dried down and autoradiographed (Section II 2K), and the autoradiograph scanned on a Joyce - Loebl scanning densitometer. Molecular weight markers are as in Fig. 28.

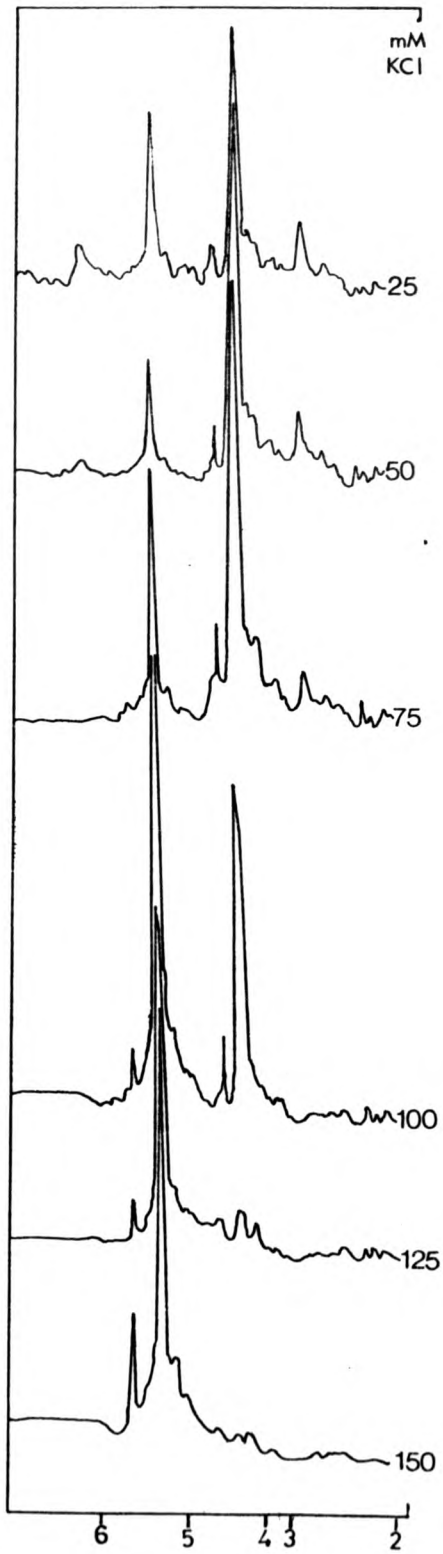

mM
KCl
25
50
75
100
125
150
6
5
4
3
2

Figure 33 Products of protein synthesis in a reticulocyte lysate programmed with chloroplast RNA with increasing potassium ion concentration.

Reticulocyte lysate incubations (20 µl) were set up as described in Section II 2E(i)a and contained 10 µg chloroplast RNA, 0.5 mM magnesium acetate and the potassium chloride concentrations indicated. Incubation was at 30° C for 60 minutes. Equal portions of each incubation were prepared for electrophoresis by the method set out in Section II 2E(i)b and analyzed by electrophoresis on a 10-30% polyacrylamide gradient Laemmli gel containing SDS (Section II 2F(i)b). The gel was dried down and autoradiographed (Section II 2K), and the autroradiograph scanned on a Joyce-Loebl scanning densitometer. Molecular weight markers are as in Fig. 28.

assay amounts of translatable mRNA for LSU and peak D, it is necessary to identify these polypeptides in the assay mixture. A convenient method of identification is the partial proteolytic digestion technique of Cleveland et al (1977). The polypeptides to be identified are electrophoresed on an SDS polyacrylamide gel and the bands cut from the wet gel. The bands are transferred to the slots of a second SDS polyacrylamide gel, and overlaid with a suitable proteolytic enzyme. The polypeptide and enzyme are electrophoresed into the stacking gel; electrophoresis is then halted for half an hour to allow digestion to proceed, and the digestion fragments separated by electrophoresis into the resolving gel. Cleveland et al (1977) used this technique successfully to obtain reproducible one-dimensional peptide maps of albumin, tubulin and alkaline phosphatase.

Figure 34 shows the peptide fragment patterns of LSU, synthesized in either a reticulocyte lysate extract (scan A) or in isolated chloroplasts (scan B), generated by digestion with S. aureus V8 protease. The qualitative pattern of bands is very similar in the two preparations, although there are obvious quantitative differences in the peaks of around 25 000 M. wt. These differences are not necessarily indicative of differences between the LSU polypeptides since the digestion is incomplete, and the band patterns may result from different digestion kinetics. The patterns of polypeptide fragments generated by S. aureus V8 protease digestion of peak D polypeptides synthesized in isolated chloroplasts (Figure 34, track C) and in a reticulocyte lysate extract (Figure 34, track D) are also qualitatively similar to one another. The patterns generated for LSU and peak D are completely different from eachother. The reticulocyte lysate therefore translates chloroplast mRNA for LSU and peak D polypeptides with fidelity and can

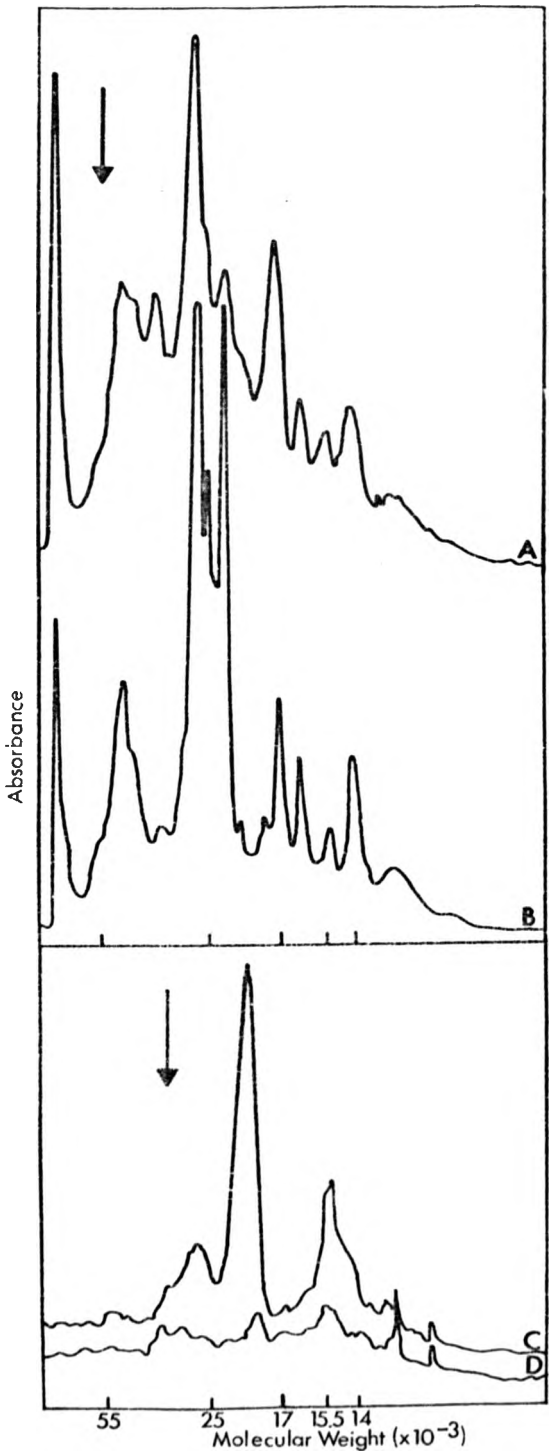

Absorbance
A
B
C
D
55
25
17
15.5
14
Molecular Weight (x10⁻³)

Figure 34 Cleveland partial protease digestion of LSU and peak D polypeptides.

Polypeptides were labelled with [^{35}S] methionine in either isolated chloroplasts (Section II 2D(ii)) or in a reticulocyte lysate cell-free system (Section II 2E(ii)) and analyzed on SDS-polyacrylamide Laemmli gels containing a 10-30% polyacrylamide gradient (Section II F(i)b). The LSU and peak D bands were chopped out and re-electrophoresed on a second gel in the presence of 0.5 µg Staphylococcus aureus V8 protease (Section II 2G). The second gel was dried and autoradiographed for 28 days (Section II 2K). Tracks were scanned on a Joyce-Loebl scanning densitometer.

A. LSU synthesized in a reticulocyte lysate extract.

B. LSU synthesized in isolated chloroplasts.

C. Peak D synthesized in isolated chloroplasts.

D. Peak D synthesized in a reticulocyte lysate extract.

be used as a quantitative assay for translatable mRNA for these two polypeptides.

C. Electrophoretic analysis of chloroplast RNA

Total chloroplast RNA prepared from developing leaves was analyzed on 2.4% polyacrylamide gels (Section II 2F(ii)) to check that the RNA from older leaves was not degraded. The chloroplast 23S rRNA (M. wt. 1.05×10^6) contains hidden breaks, which are nicks inserted at specific points along the molecules during maturation (Leaver, 1973). These nicks generate fragments of 0.87×10^6, 0.67×10^6, 0.48×10^6, 0.42×10^6 and 0.24×10^6 from the 1.05×10^6 rRNA species in spinach (Hartley and Read, 1979). The 16S rRNA (0.6×10^6 M. wt.) does not contain such breaks. Since the RNA preparation in this study (Section II 2D(iii)) was carried out at room temperature in the absence of divalent cations,(conditions which break the hydrogen bonds holding the fragments together), the 1.05×10^6 M. wt. rRNA was fragmented in all the preparations made (Figure 35). Fragments of 0.67×10^6, 0.48×10^6 and 0.42×10^6 M. wt. are visible. However, there is no other detectable degradation of the rRNA molecules. There is also no detectable contamination of the preparations by cytoplasmic rRNA s which have molecular weights of 1.34×10^6 and 0.7×10^6 (Hartley and Ellis, 1973), although contamination of a few micrograms of these species would not be visible on this gel system. This indicates that the majority of the polypeptides synthesized in the reticulocyte lysates programmed with such chloroplast RNA are likely to be derived from chloroplast mRNA s. In summary, the RNA preparations are:-

1. free of detectable cytoplasmic rRNA contaminants;
2. not obviously degraded by nucleases during extraction.

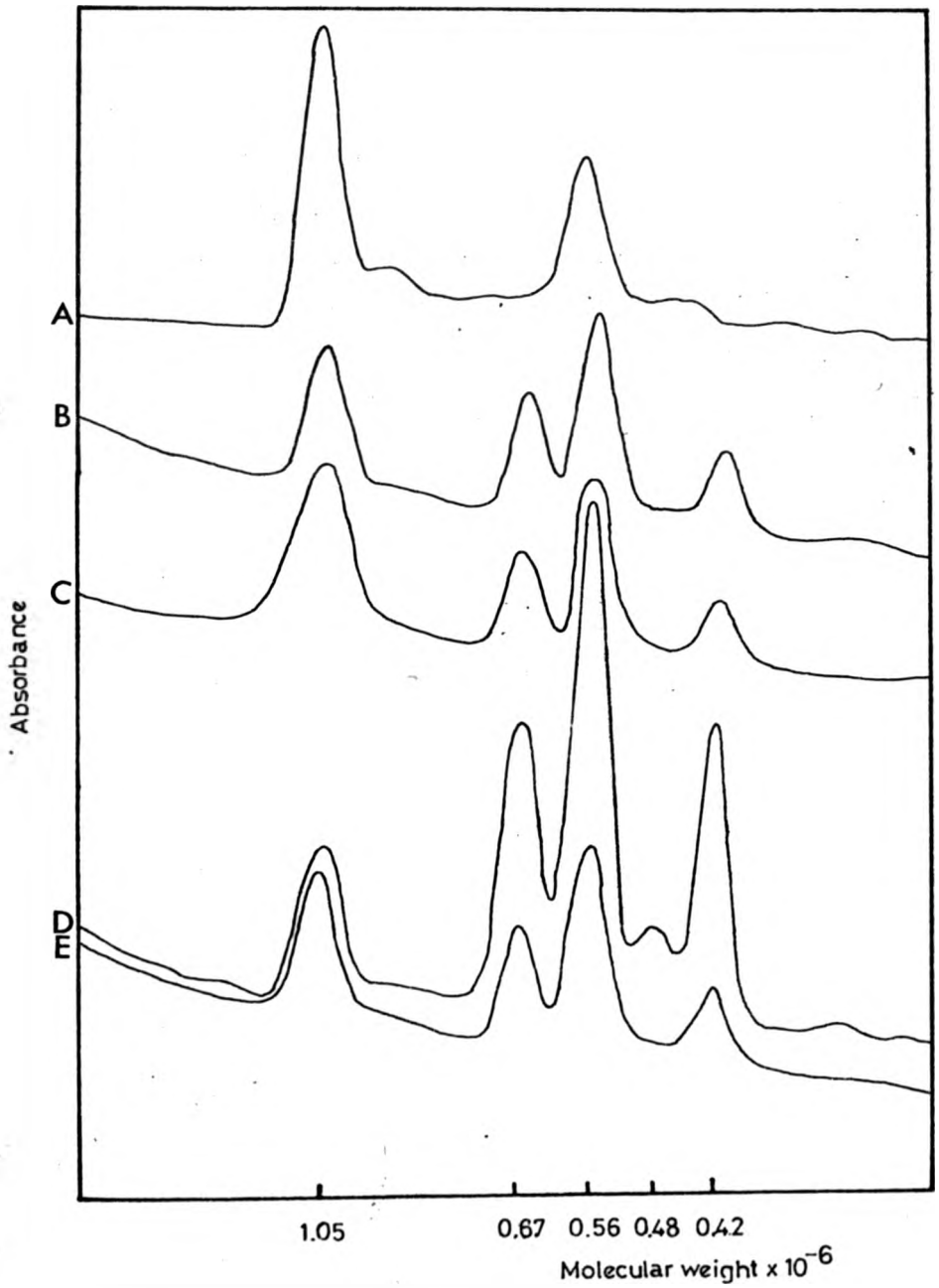

A
B
C
D
E
Absorbance
1.05
0.67
0.56
0.48
0.42
Molecular weight x 10^{-6}

Figure 35 Electrophoretic analysis of chloroplast rRNA

Chloroplast RNA was prepared by the method described in Section II 2D(iii) and analyzed by electrophoresis on 2.4% polyacrylamide gels (Section II 2F(ii)). The gels were scanned at 260 nm at a full scale deflection of 1.5 absorbance units on a Gilford linear transport scanner. Scans show rRNA regions of the gels.

A. 30 µg *E. coli* rRNA marker

B. 40 µg chloroplast RNA from 12 day leaves

C. 40 µg chloroplast RNA from 20 day leaves

D. 40 µg chloroplast RNA from 23 day leaves

E. 40 µg chloroplast RNA from 35 day leaves

D. Estimation of the ratio of incorporation of [^{35}S] methionine into LSU and peak D

The amount of translatable mRNA for LSU and peak D in chloroplasts isolated from developing leaves was assayed in a reticulocyte lysate extract. Figure 36 shows the gel pattern of polypeptides synthesized in a cell-free system programmed with chloroplast RNA prepared from leaves 19, 23, 26 and 32 days after germination. It is clear that the pattern of polypeptides synthesized throughout this period of development remains qualitatively the same. However, there is a decrease in the synthesis of LSU, and an increase in incorporation into peak D. In order to quantitate these changes, the relative incorporation into LSU and peak D was measured in two ways:-

1. by scanning autoradiographs and calculating the ratio of peak heights (Section III 2B);
2. by excising the bands from gels and counting directly their radioactivity (Section II 2H).

Both methods were used in order to check the validity of the scanning procedure; the data for quantitation from peak heights is shown in Table VI, and that from measurement of radioactivity in Table VII. There is a decrease in the ratio of LSU to peak D synthesized whether the RNA is assayed at 7.5 or at 15 µg/assay tube. These RNA concentrations lie within the range where the incorporation in the cell-free system in response to added RNA is linear (Section III 4A). The decrease in ratio calculated from scans is about four-fold when 7.5 µg RNA per assay is used, and about five-fold when 15 µg RNA per assay is used. The decrease in ratio calculated from direct counting of bands is two to three-fold. Scanning of peak heights is a less satisfactory method of measuring the ratios of LSU and peak D synthesized in vitro

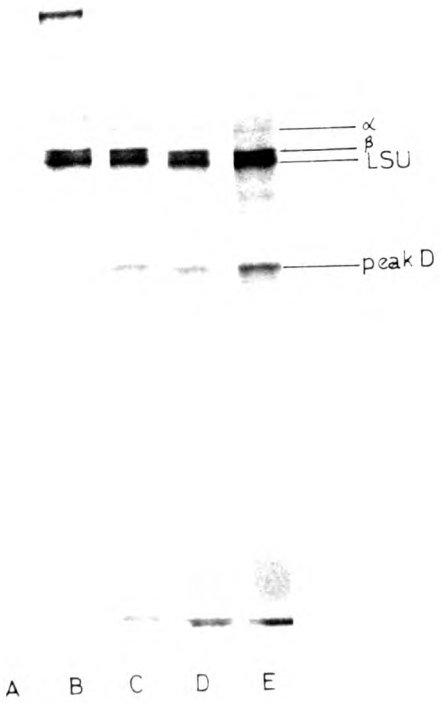

α
β
LSU
peak D
A
B
C
D
E

Figure 36 Changes in the translation products of spinach chloroplast RNA during leaf development.

Spinach chloroplast RNA was prepared (Section II 2D(iii)) at the times indicated, and assayed in a reticulocyte lysate mixture (Section II 2E(ii)) containing 7.5 µg RNA and 5 µCi [^{35}S] methionine in a final volume of 20 µl. The products of translation were analyzed on a Laemmli slab polyacrylamide gel (10-30% acrylamide concentration gradient as described in Section II 2F(i)b). The autoradiograph was exposed for 93 hours. The tracks contain equal aliquots (16 µl) of incubation mixtures.

A. No added RNA

B. 7.5 µg 19 day RNA

C. 7.5 µg 23 day RNA

D. 7.5 µg 26 day RNA

E. 7.5 µg 32 day RNA

Day after germination	Leaf length (mm)	Ratio of LSU: peak D from 7.5 µg RNA/assay	Ratio of LSU: peak D from 15 µg RNA/assay
12	9	8.3	7.5
15	19	7.8	9.2
19	26	7.5	3.7
20	33	1.9	4.8
23	39	1.8	3.5
32	51	1.6	2.4
35	50	2.5	1.7

f LSU:
from 15
assay

5

2

7

8

5

4

7

Table VI Ratios of incorporation of [^{35}S] methionine into *in vitro*-synthesized LSU and peak D as estimated from densitometer scans.

Chloroplast RNA prepared from developing leaves (Section II 2D(iii)) was assayed in a reticulocyte lysate system (Section II 2E(ii)) at a concentration of 7.5 µg or 15 µg RNA/20 µl assay tube. Aliquots (16 µl) of incubation mixtures were analyzed on Laemmli 10-30% polyacrylamide gels containing SDS (Section II 2F(ii)b), the gels dried down and autoradiographed (Section II 2K). The autoradiographs were scanned on a Joyce-Loebl densitometer, and the peak heights for LSU and peak D expressed as a ratio for each RNA concentration.

Day after germination	Leaf length (mm)	Ratio of LSU: peak D from 7.5 µg/RNA assay	Ratio of LSU: peak D from 15 µg/RNA assay
12	9	4.6	6.4
15	19	3.2	4.3
19	26	2.5	4.4
20	33	2.4	4.3
23	39	2.5	3.7
32	51	2.6	3.7
35	50	2.1	1.9

Table VII Ratios of incorporation of [^{35}S] methionine into *in vitro*-synthesized LSU and peak D, as estimated from the radioactivity in gel bands.

Reticulocyte lysate products of protein synthesis directed by chloroplast RNA were analyzed as described in Table VI. The bands of dried-down gel were analyzed for radioactivity by the method described in Section II 2H. The incorporation in to the LSU and peak D bands is expressed as a ratio.

because the peak D band is far less heavily labelled than in either *in vivo* or in isolated chloroplasts. Despite the variability in the values calculated from scans, it is clear from both methods of measurement that the synthesis of LSU relative to peak D during leaf development follows a similar time course in the reticulocyte lysate system as it does *in vivo* and in isolated chloroplasts.

E. Discussion

Chloroplast mRNA has been successfully translated in several heterologous cell-free protein-synthesizing systems. Using an *E. coli* extract, Hartley *et al* (1975) translated spinach chloroplast mRNA to yield two major products, LSU and peak D. Messenger RNA for LSU from *Chlamydomonas* has also been translated in this system (Sano *et al*, 1979; Howell and Gelvin, 1978), although an earlier study observed only incomplete polypeptides (Howell *et al*, 1977). The pattern of polypeptides directed by chloroplast RNA in these heterologous cell-free systems is dependent on the system and on the conditions employed. Bottomley *et al* (1976) could not demonstrate translation of spinach chloroplast RNA in a wheat germ extract whilst Sagher *et al* (1976) observed translation of LSU mRNA from *Euglena*. *Spirodela* chloroplast RNA can be translated in both wheat germ and *E. coli* systems, but the translation of LSU mRNA relative to the total incorporation was much higher in the *E. coli* system (Reisfeld *et al*, 1978). Variability in the translation of an mRNA for a 32 000 M. wt. thylakoid polypeptide has also been observed in these cell-free systems. The mRNA for such a polypeptide in *Spirodela* translates better in a wheat germ extract than in an *E. coli* system (Reisfeld *et al*, 1978). In maize, the mRNA for a polypeptide of this mobility is translated more efficiently in the reticulocyte lysate

system than is LSU mRNA (Coen et al , 1978). In the present study, the amount of peak D synthesized relative to the total synthesis in the reticulocyte system directed by chloroplast RNA was lower than in isolated chloroplasts from the same plants. The reasons for this relatively poor translation of peak D in this system are not clear. However, it is not a consequence of RNA concentration (Figure 30), or potassium and magnesium ion concentrations (Figures 32 and 33), since there was no marked increase in the relative amount of peak D synthesized under any of the conditions tested. It must be concluded that, under the conditions used in the reticulocyte lysate in the present study, peak D is a less efficient mRNA than in chloroplasts.

It is clear from all these studies that the choice of cell-free system depends on which polypeptides are of interest. The reticulocyte lysate is the most suitable of these systems for the quantitative assay of translatable chloroplast mRNA for the following reasons:-

1. the endogenous incorporation is low;
2. both LSU and peak D mRNA s are translated
3. there is a small proportion of prematurely-terminated polypeptides.

A 32 000 M. wt. polypeptide, possibly analogous to peak D in pea and spinach (Section I 3A) is synthesized as a precursor of 34 000 M. wt. in a reticulocyte lysate extract programmed with maize chloroplast RNA (Coen et al , 1978), and as a 33 500 M. wt. precursor in a wheat germ extract programmed with Spirodela chloroplast RNA (Edelman and Reisfeld, 1978). In the present study, there is no evidence to suggest that peak D is synthesized as a precursor, since the mobilities of the cell-free system product and the polypeptide synthesized in isolated chloroplast are closely

similar. It is possible, however, that peak D is synthesized as a precursor only very slightly larger than the mature, thylakoid-bound polypeptide.

Cleveland mapping has been used to examine the proteolytic fragment pattern of the 32 000 M. wt. polypeptides synthesized in maize (Grebanier et al , 1978) and Spirodela (Edelman and Reisfeld, 1978). Both these polypeptides yield cleavage patterns with S. aureus V8 protease which are similar, visually to those observed for peak D in this study. Unfortunately, neither of the published cleavage patterns have any molecular weight calibration, so a more direct comparison is not possible. It is clear, however, that in Spirodela the difference between the cleavage patterns of the 32 000 M. wt. polypeptide and its precursor (33 500 M. wt.) obtained with papain and S. aureus V8 protease is confined to one band.

Despite the problems of cell-free translation, it has been possible to use cell-free systems to measure amounts of translatable mRNA. Reisfeld et al (1978) have correlated an increase in a mRNA of M. wt. 0.5 x 10^6 in greening Spirodela fronds with increased synthesis of the 32 000 M. wt. thylakoid polypeptide. Similarly, Coen et al (1978) have shown that during the greening of maize etioplasts there is an increase in translatable mRNA for the 34 000 M. wt. precursor to the 32 000 M. wt. thylakoid polypeptide. The induction of phenylalanine ammonia lyase and flavonone polypeptides in irradiated parsley cells has recently been shown to be accompanied by increased amounts of translatable mRNA in reticulocyte lysate extracts (Schroder et al , 1979). In these reports and in the present study there is a good correlation between changes in protein synthesis in vivo and translatbale mRNA amounts measured in vitro.

However, the amount of translatable mRNA for LSU in light-grown cucumber cotyledons parallels the synthesis of Fraction I protein only during the first 5 days of development (Walden and Leaver, 1978). After this period, the amount of incorporation into LSU declines in an *E. coli* S30 cell-free system, whilst accumulation of Fraction I protein continues in the cotyledon. Caution must therefore be exercised in the interpretation of such data since translational controls may also be in operation *in vivo*. This point will be discussed further in Section IV.

The main conclusions from this section are as follows:-

1. LSU and peak D mRNA are translated by the reticulocyte lysate with fidelity;

2. changes in the relative synthesis of LSU and peak D *in vivo* (Section III 2) and in isolated chloroplasts (Section III 3) are mirrored by changes in the relative amounts of translatable mRNA. This correlation will be discussed in Section IV. It is not established whether this situation is a result of differential transcription, differential mRNA turnover, or from some other kind of translational control. Further approaches to this problem will be discussed in Section IV. However, it is clear that the results in this section show that two genes located in the chloroplast genome are expressed to differing extents during the normal development of chloroplasts.

SECTION IV - GENERAL DISCUSSION

IV.1 CONTROL OF CHLOROPLAST GENE EXPRESSION DURING DEVELOPMENT

The data presented in this thesis show that there are quantitative rather than qualitative changes in chloroplast protein synthesis during development. They also indicate that the changes in the rates of synthesis of LSU and peak D during development are at least partly mirrored by changes in the amounts of translatable mRNA for these two polypeptides. These points will now be discussed further.

The spectrum of polypeptides synthesized during chloroplast development in the three systems studied does not change dramatically during the period of leaf expansion. This finding contrasts with the situation observed during greening in maize, where there is loss of etioplast - specific polypeptides and the synthesis of chloroplast polypeptides dependent on light (Grebanier *et al*, 1979). During greening, the major known polypeptides whose synthesis is dependent on light are the 32 000 molecular weight thylakoid polypeptide and the chlorophyll *a*/*b* binding protein. In addition, there is the dramatic increase in the synthesis of Fraction I protein and the coupling factor α and β subunits which are already present in etioplasts, but whose synthesis is stimulated by light (Grebanier *et al*, 1979; Lockshin, 1973). In the present study, chloroplast maturation is accompanied by a decline in the synthesis of Fraction I protein and the chlorophyll *a*/*b* binding protein, but increased synthesis of peak D. Thus, in long-term studies, etiochloroplast development and more normal chloroplast development are not interchangeable. These observations support the contention that in terms of chlorophyll synthesis and CO_2 fixation, etiochloroplast development is not a direct model for the normal development of chloroplasts from proplastids (Leech, 1977).

However, the pattern of synthesis of peak D and LSU in developing etiochloroplasts is similar to that observed in developing chloroplasts; the synthesis of LSU relative to peak D declines during development in pea etiochloroplasts (Siddell and Ellis, 1975). The present study confirms and extends this initial observation.

The results presented in this thesis, especially the *in vivo* polypeptide patterns (Section III2), serve to emphasize the relatively small number of chloroplast polypeptides whose identities have been established. In particular, no function has, as yet, been ascribed to peak D or its possible counterparts in other tissues. This raises the question as to what constitutes the identification of a polypeptide. The major criteria for identification must be structural similarity and identical properties including function. In the case of peak D , the identity of the molecule described in pea (Eaglesham and Ellis, 1974) and spinach (Bottomley *et al*, 1974) with those of maize (Coen *et al*, 1978) and *Spirodela* (Edelman and Reisfeld, 1978) cannot be fully established until a function can be ascribed to these polypeptides. However, from studies so far performed and summarized in Table E, they may share some structural similarity.

No obvious role for peak D has yet been found. Although it is very hydrophobic, as judged by its solubility in chloroform-methanol (Ellis and Barraclough, 1978), it is unlikely to be related to the chlorophyll *a/b* binding protein in function. The *Chlorina* mutant of barley, which lacks the chlorophyll *a/b* binding protein and does not contain stacked grana (Thornber and Highkin, 1974) does contain peak D (Ellis and Barraclough, 1978). Peak D can be precipitated from Triton X-100 extracts of thylakoids by antisera to CF_1 (Ellis *et al*, 1978) but is not a component of CF_o (Pick and

	PLANT			
PROPERTY	Pea	Spinach	Maize	Spirodela
Location	thylakoids[1]	-	thylakoids[2]	thylakoids[3]
Molecular weight	32 000[1]	36 000[4]	32 000[2]	32 000[3,5]
Molecular weight of precursor	-	-	34 500[2]	33 500[5]
present in stainable amounts	no[1]	no[4]	yes[2]	no[3,5]
synthesis requires light	yes[6]	-	yes[2]	yes[3]
soluble in chloroform-methanol	yes[7]	-	-	-

Table E Properties of the 32 000 molecular weight membrane protein

References

1 Eaglesham and Ellis (1974).

2 Grebanier et al (1978).

3 Reisfeld et al (1978).

4 Bottomley et al (1974).

5 Edelman and Reisfeld (1978).

6 Siddell and Ellis (1975).

7 Ellis and Barraclough (1978).

Racker, 1979). The answer to the problem of the function of peak D may lie in the dependence of its synthesis on light. Since the major chloroplast components whose synthesis is light-dependent are chlorophyll and its associated binding protein(s), peak D may be involved in the regulation of photosynthesis. Such a function must involve rapid turnover and increased synthesis in older plastids. Until appropriate mutants are found, the problem of the function of this major product of chloroplast protein synthesis is likely to remain unanswered.

A major question raised by this study is what the changing ratio in the synthesis of LSU relative to peak D reflects in terms of plastid maturation. All the relevant data have been summarised in Figure 37. Both the data from *in vivo* and from translatable MRNA assays indicate that the major change in the rate of decline of LSU: peak D synthesis occurs when the leaf is about 15-20 mm in length. From Section III 1, this leaf size represents the middle of the period of rapid leaf expansion in terms of length (Fig. 2), chlorophyll content (Fig. 6) and fraction I content (Fig. 11). The visual impression of autoradiographs of proteins from tissue labelled *in vivo* during development indicates that the ratio reflects both the declining synthesis of Fraction I protein as well as the increased synthesis of peak D. Previous studies have shown that Fraction I protein is neither synthesized nor turned over significantly in mature leaves (Peterson *et al*, 1973; Dickman and Gordon, 1975; Woolhouse, 1967); the present study confirms this observation.

It is not entirely clear why the ratio of synthesis of LSU: peak D differs according to how it is measured. The disparity between assays with isolated chloroplasts and with translatable mRNA cannot be due to the method of chloroplast preparation, since

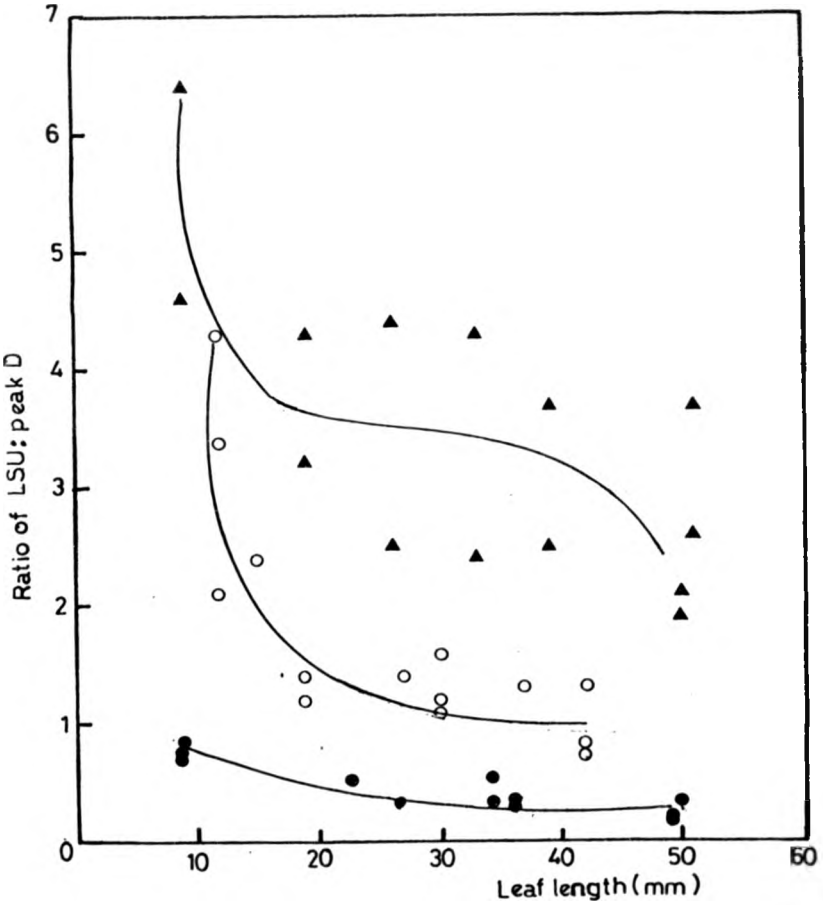

Ratio of LSU: peak D
Leaf length (mm)
0
1
2
3
4
5
6
7
10
20
30
40
50
60

Figure 37 Changes in the ratio LSU:peak D during chloroplast development.

A composite graph of data taken from Tables I, IV and VI.

o——o in vivo

●——● isolated chloroplasts

▲——▲ in a reticulocyte lysate essay

the discrepancy can be found even when the MRNA is isolated from the same preparation of chloroplasts that is used for *in vitro* protein synthesis. It is probably due, in part, to the inefficient translation of peak D mRNA in the reticulocyte lysate as compared to its translation in isolated chloroplasts. Although the reticulocyte lysate synthesizes a pattern of polypeptides which resembles, in qualitative terms, that labelled in isolated chloroplasts it additionally reflects the relative translatability of each mRNA within a mixed population of mRNA species (Nuss and Koch, 1976). Such poor translation of peak D mRNA would artificially elevate the ratio of synthesis of LSU: peak D measured by this assay procedure.

The discrepancy between the measurements made in isolated chloroplasts and those made in whole leaves is more difficult to explain. It is possible that there is a change in the available methionine pool in chloroplasts during isolation. In invoking such an explanation it is necessary to postulate that peak D is made on bound ribosomes, and that the effect of changes in the methionine pool on free and bound polysomes is different. There is no available evidence to support such a hypothesis, and it would be difficult to test.

Alternatively, chloroplasts may undergo a change in internal ion concentration during isolation which results in a change in the optima for the translation of different mRNAs. For example, a change in the potassium ion concentration could favour the translation of LSU compared to that of other mRNA species. There is some previous evidence that such ionic changes do affect protein synthesis in other systems. Thus, sodium ion concentration is involved in the shut-down

of host cell protein synthesis during picornavirus infection (Carrasco and Smith, 1976); the influx of sodium ions induced during viral infection is sufficient to inhibit host cell protein synthesis whilst not affecting the synthesis of viral polypeptides. Similarly, magnesium ion concentration can affect the relative amounts of α and β thyroid-stimulating hormone polypeptides synthesized in a Krebs Ascites extract (Chin et al, 1978). If such ionic effects are operating in isolated chloroplasts it would be difficult to demonstrate the roles of individual ions.

A third possible explanation for the low ratios of LSU: peak D synthesis measured in isolated chloroplasts is that isolation removes the chloroplast from some kind of cytoplasmic control. It is possible that factors from the cytoplasm exert positive or negative effects on chloroplast protein synthesis. This hypothesis could be tested by preparing a chloroplast protein synthesizing extract and adding back cytoplasmic fractions. Such a mechanism could be one way in which the nuclear and chloroplast protein-synthetic systems are coordinated. This type of control is consistent with the multisubunit protein hypothesis if subunits of enzymes eg. SSU are involved in protein synthetic regulation.

The main aim of the present study was to establish whether gene expression during chloroplast development is under transcriptional control. From the data obtained, this question can only be partially answered. If the synthesis of LSU and peak D is solely controlled by transcription of their genes, then it would be predicted that in a quantitative heterologous cell-free system, the changes in the ratios of LSU: peak D mRNA activity during development should mirror the ratios measured in vivo. Although the pattern of decrease in this ratio is similar in vivo and in the cell-free system, the match is not exact. Thus, although transcriptional control appears to be

involved in chloroplast gene expression, some additional translational control cannot be ruled out. For example, the relatively poor translation of peak D in the cell-free extract may reflect the lack of translational control factors operating *in vivo*. These limitations are imposed by the approach adopted for study in this project. More useful tools have since been developed, and will be discussed in Section IV 2.

The roles of transcriptional and translational control in gene expression are fundamental to many areas of cellular development in both prokaryotes and eukaryotes. The area of translational control in eukaryotes has recently been reviewed by Revel and Groner (1978) and Lodish (1976).

Transcriptional control has been postulated as the mechanism of action in the hormonal induction of a number of polypeptides; for example, in glucocorticoid induction of α-globulin synthesis in rat hepatocytes (Chen and Feigelson, 1978), prolactin induction of casein mRNA (Matusik and Rosen, 1978), hydrocortisone induction of tyrosine aminotransferase mRNA (Nickol *et al*, 1978) and estrogen induction of ovalbumin (Swaneck *et al*, 1979), and ovomucoid (Tsai *et al*, 1978). Similarly, there appears to be transcriptional control of gene expression during the light-induced synthesis of *Dictyostelium* polypeptides during germination (Giri and Ennis, 1978), procollagen synthesis in developing chick calvaria (Moen *et al*, 1979) and phosphoenolpyruvate carboxykinase synthesis in developing liver (Ruiz *et al*, 1978). However, in some cases, there may be an involvement of some form of translational control. During the induction of rat mammary gland α-lactalbumin, the mRNA and protein amounts are out of phase during the first week of induction (Nakhasi

and Qasba, 1979). Similarly, the hydrocortisone induction of the mRNAS coding for tyrosine aminotransferase and tryptophan oxygenase parallels enzyme activity, but occurs 2 hours before the peak of enzyme activity (Roewekamp et al, 1976). Such differences can be ascribed, in part, to translational control. For example, the milk protein mRNA studied by Nakhasi and Qasba (1979) may be translated more efficiently during the early stages of development. Such a mechanism may also explain the increasing synthesis of Fraction I protein during the later stages of cucumber cotyledon development, when the mRNA for LSU is declining as measured by in vitro translation (Walden and Leaver, 1978). Similarly, the induction of cellulase mRNA in pea epicotyls by 2,4-dichlorophenoxyacetic acid may be controlled by both transcriptional and translational mechanisms (Verma et al, 1975).

There are several major criticisms of the use of cell-free assays to estimate the amount of mRNA present in the situations discussed above:-

(1) Different cell-free extracts may give different spectra of products. For example, the spectrum of protamine polypeptides synthesized in the wheat germ and reticulocyte lysate extracts are different (Gedamu et al, 1979);

(2) The system may underestimate the amount of mRNA present. For example, pancreatic α-amylase mRNA measured by hybridization is between two and three-fold greater than that measured in a wheat germ cell-free system (Ray et al, 1979), perhaps because not all the mRNA is engaged in translation;

(3) The translation of mRNAS for some polypeptides is dependent on the addition of polyamines eg rabbit pancreatic α-amylase (Ray et al, 1979) and Tobacco Mosaic Virus polypeptides (Hunter et al, 1977).

To overcome such criticisms, it is necessary to use purified hybridization probes to measure mRNA sequences. In the absence of such a technique, translational assays can only give a partial answer to the question of the level of control of gene expression.

IV.2 FUTURE APPROACHES TO THE STUDY OF CHLOROPLAST GENE EXPRESSION

A major drawback of the spinach primary leaf system is that leaves of less than 5 mm in length cannot be used for biochemical studies because of the difficulty in obtaining sufficient amounts of tissue. During the major part of the present study, the spinach leaves are undergoing expansion by both cell division and cell expansion (Saurer and Possingham, 1970). In spinach secondary leaves, the plastid number per cell increases during the period of leaf expansion by division of grana-containing plastids rather than by the division of proplastids (Possingham and Saurer, 1969). Thus, the developmental sequence under study involves the development of plastids by division of mature plastids rather than by proplastid development. Thus, the observation that there are quantitative rather than qualitative changes in protein synthesis during spinach chloroplast development may be a consequence of the system chosen for study.

In order to study chloroplast development from proplastids, it is necessary to use a more synchronous and compartmentalized system than that provided by spinach. Cereal leaves provide such a system. The leaves of cereal plants grow from the base, so that the cells from base to tip form a developmental sequence (Robertson and Laetsch, 1974; Boffey et al, 1979; Leech et al, 1973). If the tissue is grown under controlled light conditions it is possible to obtain areas of leaf corresponding to defined developmental stages (Boffey

To overcome such criticisms, it is necessary to use purified hybridization probes to measure mRNA sequences. In the absence of such a technique, translational assays can only give a partial answer to the question of the level of control of gene expression.

IV.2 FUTURE APPROACHES TO THE STUDY OF CHLOROPLAST GENE EXPRESSION

A major drawback of the spinach primary leaf system is that leaves of less than 5 mm in length cannot be used for biochemical studies because of the difficulty in obtaining sufficient amounts of tissue. During the major part of the present study, the spinach leaves are undergoing expansion by both cell division and cell expansion (Saurer and Possingham, 1970). In spinach secondary leaves, the plastid number per cell increases during the period of leaf expansion by division of grana-containing plastids rather than by the division of proplastids (Possingham and Saurer, 1969). Thus, the developmental sequence under study involves the development of plastids by division of mature plastids rather than by proplastid development. Thus, the observation that there are quantitative rather than qualitative changes in protein synthesis during spinach chloroplast development may be a consequence of the system chosen for study.

In order to study chloroplast development from proplastids, it is necessary to use a more synchronous and compartmentalized system than that provided by spinach. Cereal leaves provide such a system. The leaves of cereal plants grow from the base, so that the cells from base to tip form a developmental sequence (Robertson and Laetsch, 1974; Boffey et al, 1979; Leech et al, 1973). If the tissue is grown under controlled light conditions it is possible to obtain areas of leaf corresponding to defined developmental stages (Boffey

et al, 1979). Protein synthetic studies and in vitro assay of mRNA prepared from such sections could yield information on the expression of chloroplast genes during development of proplastids.

Such a study relies on suitable methods for the isolation of proplastids and the assay of protein synthesis in vitro, as have been developed for chloroplasts isolated from pea (Blair and Ellis, 1973) and spinach (Bottomley et al, 1974). Proplastids have been isolated from maize, and shown to incorporate isotope into fatty acids (Hawke et al, 1974). Recently, Euglena proplastids have also been purified and characterised (Dockerty and Merrett, 1979). It may be possible to use such plastids for in vitro protein synthesis. Fractionation of leaf homogenates on Percoll gradients of the kind described by Mendiola-Morgenthaler et al (1976) should yield intact plastid preparations suitable for such a purpose.

The major advances in the chloroplast field since the start of the present study have included the successful cloning and identification of several chloroplast genes. The gene for the large subunit of fraction I protein has been cloned and identified in maize (Bedbrook et al, 1979), spinach (Bottomley et al, 1978) and Chlamydomonas (Malnoe et al, 1979). Apart from allowing the mapping of chloroplast genes onto the DNA circle (Section I2B), these clones are useful tools for the analysis of gene expression during chloroplast development.

The maize LSU gene clone has already been used to examine the control of gene expression in the dimorphic chloroplast types of the C4 plant maize (Link et al, 1978). The bundle sheath chloroplasts contain Fraction I protein whereas the mesophyll chloroplast do not. Using the LSU gene probe, Link et al have shown that although both types of chloroplast contain the LSU gene, only the bundle sheath

cells contain hybridizable mRNA. The differential expression of this gene in the two types of chloroplast is also observed at the level of translatable mRNA. Thus, in the development of maize chloroplasts, there is differential gene expression at the level of mRNA availability, although it is not clear yet whether this is a result of differential transcription or of differential RNA turnover Such an approach can be extended to cover the expression of chloroplast genes during chloroplast differentiation in other systems.

Recently, a procedure has been devised to bind RNA to a paper support (Noyes and Stark, 1975). If RNA prepared from different stages of chloroplast development is fractionated on an agarose gel (McMaster and Carmichael, 1977) it can be transferred to such paper and the paper subsequently hybridized to a series of known labelled chloroplast DNA gene fragments. In order to detect hybridization, the probe has to be labelled; one procedure is to nick-translate the DNA in the presence of α-[^{32}P] triphosphates (Rigby et al, 1977). The major advantage of this procedure is that the RNA-paper complex can be re-used so that as more chloroplast genes become available, one RNA preparation could be tested for several sequences. This procedure is also potentially quantitative, and could be used to estimate the amount of mRNA for specific chloroplast polypeptides present in developing chloroplasts.

An alternative procedure for the identification of sequences expressed during chloroplast development involves immobilizing the cloned DNA on filters and hybridizing the RNA preparations to this probe (Harpold et al, 1978). The specifically bound RNA can be eluted and translated in a cell-free system, and the products

identified by partial proteolytic digestion (Cleveland et al, 1977) or by immunological techniques.

The number of studies of chloroplast gene expression during development which uses these newer techniques is limited. Using the cloned gene for the 32000 molecular weight thylakoid polypeptide, Bedbrook et al (1978) have shown that the synthesis of the mRNA for this polypeptide is dependent on light in maize. The mRNA hybridizing to the cloned probe, and translatable mRNA for this polypeptide, appear simultaneously. As in the case of LSU gene expression, it is not known whether the control of messenger availability is at the level of transcription or of RNA turnover.

A fundamental problem remaining is the identification of chloroplast genes on the DNA circle. To date, only LSU and the 32 000 molecular weight membrane polypeptide have been mapped onto chloroplast DNA (Section I2B). Since there are other polypeptides which are synthesized by isolated chloroplasts (Section I2C) it is reasonable to assume that these too are encoded in chloroplast DNA. These polypeptides should provide further probes for chloroplast gene expression during development.

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